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CAUSES OF NEONATAL MORTALITY

IN THE NEW ZEALAND SEA LION (*PHOCARCTOS HOOKERI*)

A Thesis

presented in partial fulfilment of

the requirements for the degree of

Doctor of Philosophy

in

Veterinary Pathology

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Abstract

CAUSES OF NEONATAL MORTALITY IN THE NEW ZEALAND SEA LION (*PHOCARCTOS HOOKERI*)

As part of a health survey of New Zealand sea lions (*Phocarctos hookeri*) on Enderby Island, Auckland Islands (50° 30'S, 166° 17'E), neonatal mortality was continuously monitored at the Sandy Bay Beach rookery, from 1998/1999 to 2004/2005. The primary causes of death were categorised as trauma (35%), bacterial (24%) and hookworm (13%) infections, starvation (13%) and stillbirth (4%). During the 2001/2002 and 2002/2003 breeding seasons, bacterial epidemics caused by *Klebsiella pneumoniae* increased mortality by three times the mean in non-epidemic years.

Uncinaria spp. from New Zealand sea lion (NZSL) pups was described for the first time using morphometric criteria. It differed from the two species already described in pinnipeds, *Uncinaria lucasi* and *Uncinaria hamiltoni*, suggesting the existence of a different morphotype in NZSLs. A study on the epidemiology of hookworm infection showed that all pups up to at least three months of age harboured adult hookworms in their intestines and transmammary transmission was identified as the route of infection of NZSL pups. Uncinariosis as a primary cause of mortality was generally associated with anaemia, haemorrhagic enteritis and frank blood in the lumen. The relationship between hookworm burden and clinical disease could not be clearly established.

The 2001/2002 and 2002/2003 bacterial epidemics at Sandy Bay Beach rookery were caused by a clonal strain of *Klebsiella pneumoniae* as verified by pulse-field gel electrophoresis and antimicrobial testing. Suppurative arthritis was the most common post-mortem diagnosis during the two epidemic seasons. Internal lesions were consistent with septicaemia, which explained the wide range of organs from which the pathogen was grown in pure culture. A serological test investigating the exposure of NZSLs to *Klebsiella* spp. showed that the large majority of pups up to two months of age did not have any anti-*Klebsiella* antibodies, even after the epidemics, but that almost all the adults were seropositive. In addition, passive immunoglobulin (Ig) transfer from lactating females to neonates was examined by measuring IgG levels in pups and was very low compared to terrestrial mammals although similar to other pinniped neonates.

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Preamble

The research reported in this thesis aimed at identifying and pondering the causes of neonatal mortality in New Zealand sea lion (NZSL) (*Phocarctos hookeri*) pups at Sandy Bay Beach on Enderby Island (New Zealand's Sub-Antarctic Islands, between 48ºS and 53ºS). Investigation into neonatal mortality commenced in 1998/1999 after an unusual mass mortality of NZSLs was noted during the 1997/1998 breeding season. A variety of personnel were involved in undertaking necropsies and collection of samples up until the commencement of these PhD studies in 2003/2004. However analysis of all these samples and consequent data are part of this PhD research. The thesis is articulated around two major themes, hookworm infection and bacterial epidemics caused by *Klebsiella pneumoniae*. These were investigated in the course of parallel studies conducted both in the field and in several laboratories on mainland New Zealand. Therefore the thesis appears as a succession of discrete chapters that have been, or will be, submitted to various journals for publication. To facilitate some uniformity of presentation, all chapters have been presented here in the same style and format. Where applicable, cross-referencing to other chapters or sections in chapters has also been included. A set of complementary documents including the standard operating procedures developed and used for this research is provided for each chapter in the Appendices section. These are numbered as for the chapter they refer to and are generally in addition to the documents presented to the various journals for publication and are also referred to in the relevant section of these chapters. A list of abbreviations commonly used in the text is presented immediately before the literature review.

A detailed literature review of the causes and circumstances of mortality in neonates of other pinniped species than the New Zealand sea lion is given in Part One (Chapter 1). Part Two includes Chapter 1 that presents the causes of neonatal mortality observed at Sandy Bay Beach for seven consecutive years. Prevalence of the main causes of death and necropsy findings are detailed in this section. Part Three is dedicated to studies on hookworm infections in New Zealand sea lion pups. It comprises Chapters 3 to 6. The first

chapter gives a morphometric description of the species found in NZSLs and compares it to the hookworm species occurring in otariids around the world. This has been published in Parasitology Research (Appendix 3.1). A morphological description of larval stages and immature adults can be found in Appendix 3.2 as a series of drawings and microscopic pictures. This supplement provides some important and innovative data on the anatomical development of the parasite in its immature forms. Chapter 4 consists of a brief study on the weather conditions at Sandy Bay Beach and the possible influence of temperature fluctuations on hookworm free-living stages. Chapter 5 considers the life cycle and the descriptive epidemiology of hookworms in NZSLs. Chapter 6 describes the clinical pathology associated with this parasitic infection. It also investigates its effects on some biological parameters of pups. Part Four includes three chapters describing the 2001/2002 and 2002/2003 *Klebsiella pneumoniae* epidemics on the Auckland Islands. The bacterial agent is first characterized using phenotypical and molecular analyses (Chapter 7). Then some aspects on the epidemiology and clinical pathology of these epidemics are investigated (Chapter 8), followed by a chapter on a serological survey covering 7 years of population monitoring at Sandy Bay Beach (Chapter 9). A general discussion concludes this study on neonatal mortality in the New Zealand sea lion (Part Five, Chapter 10).

The elements of histopathology associated with *Klebsiella pneumoniae* epidemics and presented in Chapter 8 correspond to the research achieved within the frame of this PhD thesis. However, further analyses are recommended before submitting this work for publication in a scientific journal. Other experimental chapters have been or will be submitted for publication as follows: *Journal of Wildlife Diseases* (Chapter 2); *Parasitology Research* (Chapter 3, published; Chapter 5); *Journal of Comparative Pathology* (Chapter 6) and *Veterinary Microbiology* (Chapters 7 and 9).

List of Abbreviations

Ab…….Antibody

Ag ……Antigen

BA ……....Blood Agar

CPE ……..Cythopathogenic Effect

DOC……..Department Of Conservation (New Zealand)

ELISA……...Enzyme-Linked ImmunoSorbent Assay

ENSO……....El Niño Southern Oscillation

ESBL…….Extended Spectrum Beta-Lactamases

G.A.L.T…….Gut-associated Lymphoid Tissue

Gram+/Gram-……..Gram stain positive/ negative

Hct ……...Hematocrit

H&E ……... Hematoxylin & Eosin

Ig ……. Immunoglobulin

IgG …….. Immunoglobulin G

L_1, L_2, L_3……...Larvae first-stage, second-stage and third-stage

LPS……...Lipopolysaccharides

MAC…….MacConkey agar

MHC…… Major Histocompatibility Complex

MGG……May-Grunwald Giemsa

MIC……..Minimum Inhibitory Concentration

NZSL ……...New Zealand sea lion

PCR……..Polymerase Chain Reaction

PFGE ……...Pulsed-Field Gel Electrophoresis

RBC …….Red blood cell

SOP……...Standard Operating Procedure

WB……Western Blot

WBC ……White Blood Cell

XLD …….Xylose-lysine desoxycholate agar

Most frequently cited Pinniped species

Otariids

New Zealand sea lion……*Phocarctos hookeri*

New Zealand fur seal…….*Arctocephalus forsteri*

Australian sea lion……..…*Neophoca cinerea*

Australian fur seal…….*Arctocephalus pusillus doriferus*

Northern fur seal……*Callorhinus ursinus*

California sea lion……..*Zalophus californianus*

Steller's sea lion…….*Eumetopias jubatus*

Antarctic fur seal…….*Arctocephalus gazella*

Sub-Antarctic fur seal…….*Arctocephalus tropicalis*

Juan Fernandez fur seal……..*Arctocephalus philippii*

South American sea lion……*Otaria flavescens* (=*O. byronia*)

South American fur seal…….*Arctocephalus australis*

Galapagos fur seal……..*Arctocephalus galapagoensis*

South African fur seal…….*Arctocephalus pusillus pusillus*

Phocids

Grey seal……*Halichoerus grypus*

Harbour seal……...*Phoca vitulina*

Crabeater seal…….*Lobodon carcinophagus*

Northern elephant seal……*Mirounga angustirostris*

Southern elephant seal……*Mirounga leonina*

Chapter 1

General Introduction and Literature Review

Introduction

The New Zealand sea lion (NZSL), *Phocarctos hookeri*, is a species endemic to New Zealand which has been weakened by a declining population size, due to bacterial epidemics and food competition from the squid fishery around their breeding colonies on the Auckland Islands. A continued increase in trawl net fishing, ecotourism and other human activities represent some potential risks for the sea lions. Their population had already shown a dramatic decrease after sealing campaigns were conducted during the 19th century on the Auckland Islands, their main breeding site. Despite being protected by international and New Zealand legislation, the NZSL population is declining and its ecology and health status are uncertain. This review outlines the information available on NZSLs, and includes literature on neonatal biology and mortality of pinnipeds, with an emphasis on infectious agents and the immune system of pups to underpin the research described in subsequent chapters.

I. The Hooker's or New Zealand sea lion (NZSL), *Phocarctos hookeri*

I. 1. Pinnipeds, otariids and sea lions

Seals, sea lions and walruses belong to a very diverse group of marine carnivores: the pinnipeds (order Pinnipedia). This includes three families: the Otariidae or eared seals (sea lions and fur seals), the Phocidae or true (earless) seals (seals and elephant seals) and the Odobenidae or walruses (Riedman, 1990).

The New Zealand sea lion (NZSL) (*Phocarctos hookeri*, also known as Hooker's sea lion) is now one of the world's rarest pinnipeds but historical records have shown that this species was abundant around mainland New Zealand and in the adjacent sub-Antarctic region before human activity considerably reduced their population.

Figure 1.1 New Zealand or Hooker's sea lion bull (foreground) and females on Dundas Island (Auckland Islands).

Figure 1.2 New Zealand sea lion pup at Sandy Bay Beach on Enderby Island (Auckland Islands).

The other otariid species reported in this region is the New Zealand fur seal, *Arctocephalus forsteri*. There are only small numbers of New Zealand furs seals on Enderby Island and they are not breeding colonies. Fur seals do not share the same locations and have never been seen within a NZSL breeding colony.

I. 2. The Auckland Islands

The New Zealand sub-Antarctic domain includes five groups of islands located between 45-55ºS and 160-185ºE. These are the Snares, Auckland, Bounty, Antipodes and Campbell Islands (Fig.1.3). They are all included in the New Zealand Exclusive Economic Zone (Fig. 1.4) and are under the management of the Southland Conservancy of the Department of Conservation (DOC). The Auckland Islands comprise a group of small islands clustered around the larger main island, Auckland Island, which is 50,990 ha in area and 40 kilometres long (Fig. 1.3) (Peat, 2003). The smaller islands include Enderby, Disappointment and Dundas Islands (Fig. 1.4). Climatic conditions on these islands are characterised by persistent westerly winds and abundant precipitation throughout the year (1,200-1,500 mm per year). Maximum air temperature ranges between 10-16ºC in the summer and between 4-10ºC in the winter. Enderby Island usually has the finest and warmest weather of all the sub-Antarctic islands (Peat, 2003).

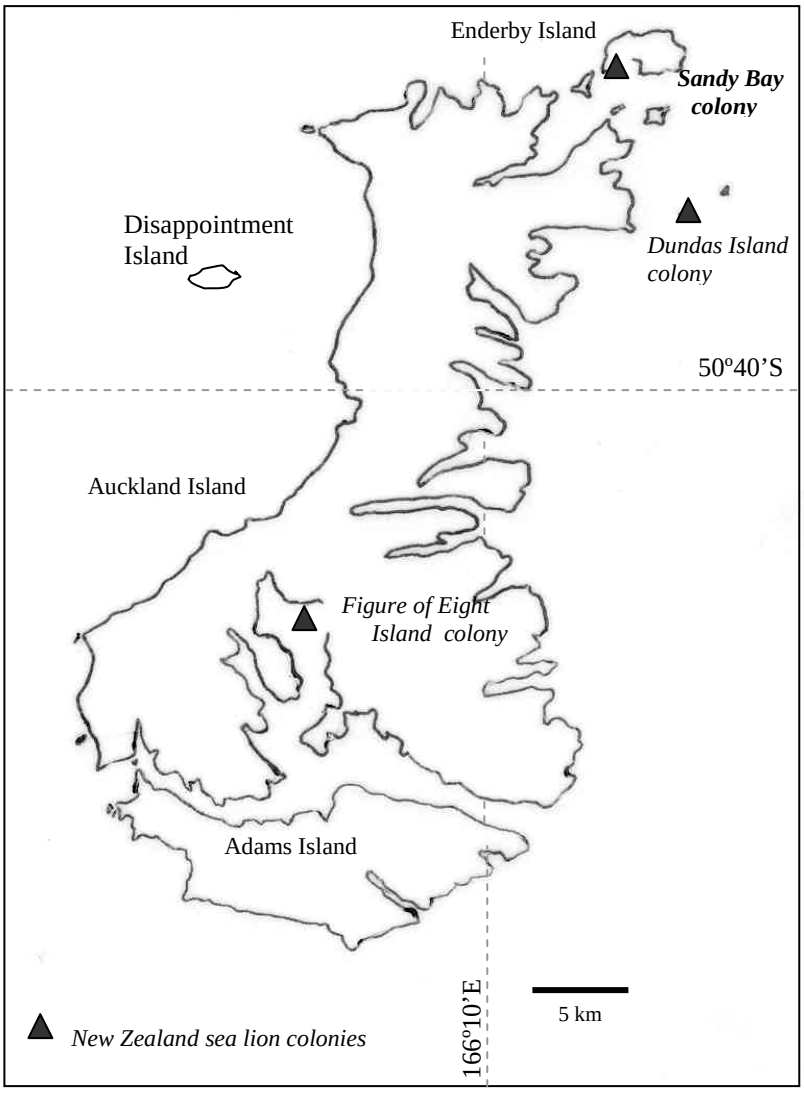

Figure 1.3 Map of the Auckland Islands showing the three colonies of New Zealand sea lions: Sandy Bay beach (Enderby Island), Dundas Island and Figure of Eight Islands.

The vegetation consists of a unique group of endemic species. This requires DOC to maintain a high level of vigilance to ensure that visitors do not disturb the biological balance by introducing any new plant species. The Auckland Islands have the southernmost forest of the Sub-Antarctic region, dominated by the Southern Rata, *Metrosideros umbellata*. On Enderby Island, three general strata can be observed. The lowest zone is the sandy beach, leading into a forest of tree ferns and Ratas (*Cyathea smithii*) between 50 and 150 metres above sea level. Above this, is a grassland of tussock and megaherbs (including *Stilbocarpa* spp. and *Pleurophyllum* spp.) (Peat, 2003).

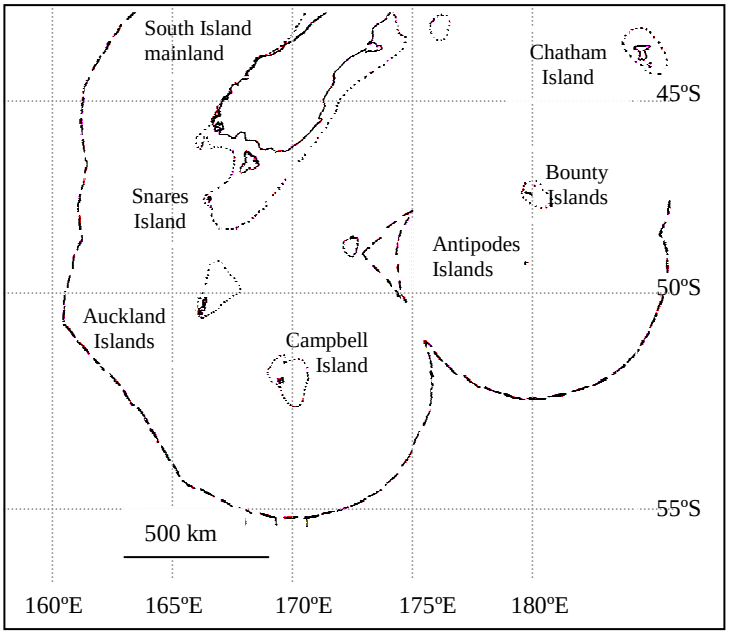

Figure 1.4 Map of the sub-Antarctic islands showing the New Zealand Economic Exclusive Zone (- - - -) and the 200 m depth contour (……).

I.3. "Seal" hunting and squid fisheries

In the early nineteenth century a sealing era began on the Auckland Islands. Hunters, mainly Australians, initially decimated the New Zealand fur seal population (*Arctocephalus forsterii*) for their skins. After the fur seal stocks were depleted, they then killed NZSLs for their oil (Childerhouse and Gales, 1998). Within a decade, both populations were severely reduced and there were few pinnipeds remaining in the area. A second campaign of hunting by whalers, the Maori and castaways from shipwrecks (Fig. 1.5, 1.6) aggravated the general reduction of the NZSL and the New Zealand fur seal populations on the Auckland Islands. In 1893, sealing for both species was prohibited by a New Zealand law. Consequently, during the twentieth century, the NZSL population showed signs of recovery (Childerhouse and Gales, 1998).

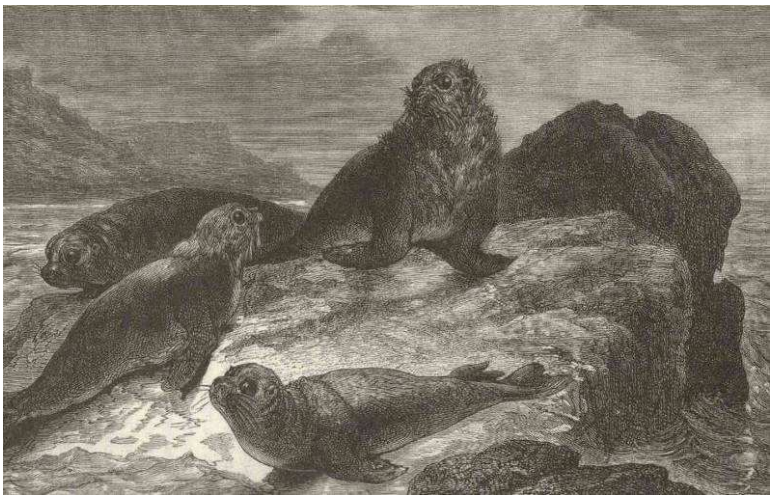

Figure 1.5 "Sea lion and young". Gravure from "Wrecked on a reef or twenty months in the Auckland Isles" by Raynal (1880).

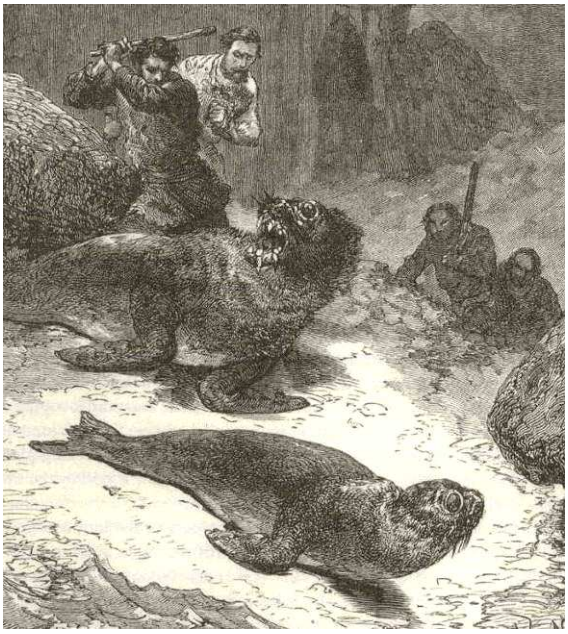

Figure 1.6 Castaways hunting a female NZSL and its pup. Gravure from "Wrecked on a reef or twenty months in the Auckland Isles" by Raynal (1880).

In the late twentieth century, the NZSL population size has been estimated to be between 12,000 and 15,000 animals, comprising approximately 7,000 sexually mature animals (Gales and Fletcher, 1999; Wilkinson et al., 2003) making it one of the smallest population sizes reported for an otariid species. Data available from that time suggested that the population has remained static for at least the last 27 and possibly 40 years (Taylor, 1971, Wilkinson et al., 2003). However, the latest estimates of pup production (Chilvers et

al., 2006b) and of the entire NZSL population (10,000 to 12,000 animals, including 5,000 mature individuals; Chilvers, unpublished data) were lower than reported before, which indicates that the NZSL population on the Auckland Islands is currently declining.

Another pressure on the NZSL population occurred with the development of a trawling fishery for arrow squid (*Notodarus sloanii*) on the Auckland Islands shelf in the 1970s. Soviet, Japanese, Korean, German, Norwegian and New Zealand trawlers contributed to annual catches of 8,000 to 34,000 tonnes of squids. The opening of the squid fishery on the 1st of February each year overlaps with the second month of the NZSL breeding season. At this time, lactating females carry out numerous foraging trips to feed their newborn (Chilvers et al., 2005b, 2006a), which puts them at risk of being caught in the trawl nets..

Despite the existence of a fishing exclusion zone around the Auckland Islands established by the Ministry of Fisheries in 1982, incidental bycatch of NZSLs by squid trawlers continues to occur. Each season between 17 to 140 sea lions, mostly adult females, are reported dead by independent observers onboard these fishing vessels (Baird, 1996; Wilkinson et al., 2003). In an attempt to limit the impact of this squid fishery on the NZSL breeding population, the New Zealand government has declared an annual quota of sea lions that can be accidentally killed before the squid fishery must close. Before 2003, this boundary had been calculated using a maximum allowable fishery-related mortality (MALFiRM, now called fishing related mortality level or FRML) based on the population estimates derived from the Gales and Fletcher (1996) model. For the past four years, FRML has been based on a Bayesian population model that sets a different quota every year by computing annual pup production estimates and other calculated population parameters (Chilvers et al., 2006b).

As a result of intensive sealing ashore and more recently accidental catch of sea lions at sea, the NZSL was classified as “vulnerable” by the International Union for the Conservation of Nature and Natural Resources (IUCN) (Reijnders et al., 1993) and “threatened” due to range restriction under the New Zealand Marine Mammals Protection Act 1978 and the New Zealand Threat Classification Status (Hitchmough, 2002).

I. 4. Breeding colonies on the sub-Antarctic islands

NZSLs breed exclusively in the sub-Antarctic islands (Gales and Mattlin, 1997); 95% of pupping occurs on the Auckland Islands (50º S 166 º E) and a small breeding colony is found on Campbell Island, 52º 33' S 169 º09' E (Childerhouse et al., 2005). In addition a few pups have been seen on Snares Island (48º S 166 º20' E) and occasionally on the Otago peninsula, South Island of New Zealand (Gales and Fletcher, 1999). There are four pupping sites on the Auckland Islands: Sandy Bay Beach (50° 30'S, 166° 17' E) and South East Point on Enderby Island (50° 30'S, 166° 19'E), Dundas Island (50° 35'S, 166° 19'E) and Figure of Eight Island (50° 46'S, 166° 01' E). These breeding sites have been monitored annually since 1994/1995. With about four times more NZSLs than at the Sandy Bay Beach rookery (Enderby Island), Dundas Island is the largest breeding colony (Wilkinson et al., 2000).

I.5. Wildlife on Enderby Island

The number of scientific expeditions to describe and count the species of fauna and flora in the sub-Antarctic region has increased considerably through the twentieth century. There is a huge abundance and diversity of birds in this area. Five seabird species including the Southern Royal Albatross (*Diomedea epomophora*) breed exclusively on the Auckland Islands. Other bird species present on the Auckland Islands include the Gibson's albatross (*Diomedea gibsoni*), the yellow-eyed penguin (*Megadyptes antipodes*) and the Auckland Island shag (*Leucocarbo colensoi*). Waters around the Auckland Islands are home to the Southern Right whale (*Eubalaena australis*) during the austral winter. Pinnipeds hauling out on Enderby Island include the NZSL, small numbers of New Zealand fur seals (*Arctocephalus forsteri*), and occasionally the southern elephant seal (*Mirounga leonina*). Since the early 1990s, there has been a significant involvement of researchers collecting information on wildlife on all groups of sub-Antarctic islands and only more recently on NZSLs on Enderby and Dundas Islands.

I. 6. The 1997/1998 epidemic season

In 1997/1998, a mass mortality event killed 60% of NZSL neonates and many adults on all three Auckland Island rookeries (North Auckland Islands, Dundas and Figure of Eight Islands). Although *Campylobacter* sp. was isolated from a limited number of samples, it was not possible to attribute the mortality to a single causative agent (Baker, 1999). This event was the first evidence that disease played a role in the demography of the NZSL. It also highlighted the lack of base-line health data on the species and provided the impetus for a directed survey of neonatal mortality in the Sandy Bay Beach colony, the second largest and most intensively studied breeding site for NZSLs.

II. Neonatal mortality in pinnipeds

In pinnipeds, pup production, growth rate and survival of both offspring and juveniles are commonly used to estimate individual fitness and population health. However, since these parameters are highly variable within and between species and years, only long-term studies can indicate realistic population trends.

II. 1. Pup production

The estimates of pup numbers have been based on two to five replicate counts by each of two to four observers. A census at South East Point on Enderby Island and Figure of Eight Island was undertaken using direct counts given the small size of the colony, while at Sandy Bay Beach on Enderby Island and at Dundas Island, the estimation was made using a mark-recapture method (Gales and Fletcher, 1999; Wilkinson et al., 2003). Mark-recapture is commonly used to estimate the abundance of threatened species (White and Burnam, 1999; Lettink and Armstrong, 2003), provided appropriate assumptions are met (Chilvers et al., 2006b). The technique used on these islands consists of identifying pups by temporarily gluing coloured plastic caps on their head, which facilitates the re-sighting of marked pups

24 hours later (Fig. 1.7). Knowing the exact number of caps used and the approximate number of marked pups re-sighted, it is possible to calculate a correction factor to apply to the total count of pups present in the colony. Pup production is defined as the estimate of live pups present in the colony plus the number of pups that have died up until the date of the count (Gales and Fletcher, 1999; Wilkinson et al., 2003; Chilvers et al., 2006b).

Figure 1.7 Mark-recapture on Dundas Island: pup with a head-cap. The arrow points at the plastic cap used to identify pups.

II.2. Survival probability of pups

Otariid pups are not weaned until four to 12 or more months, which is in contrast with phocids for which the lactation period does not exceed 45 days (Oftedal, 2000). From birth to weaning, pups are periodically left unattended on the rookery while lactating females are at sea. Consequently they are more at risk of natural causes of mortality during these absences. Survival of pre-weaned otariid pups is a very complex parameter to assess given the number of life-threatening factors surrounding birth and associated with rearing. The overall survival of a given pup cohort is estimated using resighting data. However, from

two to three months of age, NZSL pups start leaving the rookery for short periods. From this time, it becomes more difficult to assess if they are away foraging, or if they have died at sea. Therefore the acceptable duration of foraging trips has to be set prior to the survival study. There are only a few studies investigating pre-weaning survival in otariid pups.

In some otariid species, heavier birth weight has been associated with better early survival (from birth to two months of age). Heavier pups at birth showed a better early survival in sub-Antarctic fur seals, *Arctocephalus tropicalis* (Georges and Guinet, 2000; Chambellant et al., 2003) and in northern fur seals, *Callorhinus ursinus* (Boltnev et al., 1998). In New Zealand fur seals, males were born heavier than females and were shown to survive better (Bradshaw et al., 2003). Although NZSL males were also usually born heavier, they were not observed to survive better than females (Chilvers et al., 2006b). Likewise, in some other otariid species, pup gender did not seem to affect the survival probability (Boltnev et al., 1998; Georges and Guinet, 2000). Indeed, for some otariids and phocids, the reverse trend has been observed and males were reported to be more at risk than females (DeVilliers and Roux, 1992; Hall et al., 2001; Bradshaw et al., 2003). Although their study on growth and survival of NZSL pups was conducted during two seasons of high neonatal mortality due to bacterial epidemics, Chilvers et al. (2006b) found high survival probabilities in a group of pups from birth to two months of age (0.75 in 2001/2002 and 0.92 in 2002/2003). However a more extended study including non-epidemic seasons should provide a more acurate estimate in normal conditions. Regardless of gender and weight, survival of otariid pups during the first two months was generally higher than for older pups until one year of age (Boltnev et al., 1998; Chambellant et al., 2003). In this, later counts (from two months of age to weaning) may give a better indication of the annual pup survival. Unfortunately survival success for NZSLs over this period is not known. One factor that has been observed to contribute to differences in survival was the variable density on the rookery (Harcourt, 1992). For example, survival of Antarctic fur seal (*Arctocephalus gazella*) pups varied between 69% (high population density) and 97% (low density) (Doidge et al., 1984).

III. Maternal attendance and pup survival

Lactation patterns vary between pinniped species. While phocid pups suckle their mother for a short time (4 to 45 days: Oftedal et al., 1987), otariid females generally spend from four to 12 months nursing their offspring (Gentry and Kooyman, 1986; Oftedal, 2000). Duration of lactation in the NZSL is intermediate between that for otariid species which breed at high latitudes such as the Antarctic fur seal and those breeding at temperate latitudes such as the Australian sea lion (*Neophoca cinerea*) (Riet-Sapriza, unpublished data). Until they are weaned, pups are largely dependent on a milk diet provided by their dam. Milk composition varies during the lactation period but otariid species, including the NZSL, generally produce low energetic milk compared to phocids (Oftedal, 2000; Riet-Sapriza, unpublished data).

All otariids display similar lactation patterns (Oftedal, 2000). Females arrive on the rookery to give birth and stay ashore for five to nine days post-partum. Lactating females then start a cycle of foraging trips to sea alternating with nursing onshore (Oftedal, 2000; Soto et al., 2004; Chilvers et al., 2005b, 2006a). The duration of these trips is highly variable between and within otariid species and ranges from 3 to 28 days (Gentry and Kooyman, 1986; Georges and Guinet, 2000). For instance, time spent at sea by lactating females from a declining population of Steller's sea lions (*Eumetopias jubatus*) was shorter than in a stable population, being 9 and 25 hours, respectively (Davis et al., 2006). NZSL lactating females spend about 53 hours at sea and 31 hours ashore (Chilvers et al., 2006a).

During seasons of low productivity of the marine ecosystem, fur seal and sea lion females tend to extend their foraging trips, therefore increasing periods of intermittent fasting of pups staying ashore (Ono et al., 1987; Lunn and Boyd, 1993; Georges et al., 2000). For the past few decades, stochastic events such as El Niño Southern Oscillations (ENSOs) have been suggested to have had devastating effects on neonatal otariids. These effects have been noted to include decreased pup growth and a dramatic increase in neonatal mortality in South American sea lions (*Otaria flavescens*), Galapagos fur seals

(*Arctocephalus galapagoensis*) and California sea lions (*Zalophus californianus*) (Francis and Heath, 1991; Trillmich and Dellinger, 1991; Soto et al., 2004).

IV. Causes of mortality in pinniped pups

An understanding of the natural history of a species of interest is needed when investigating causes of disease or death in marine mammals. Due to their mode of life, pinnipeds face pathogens from both terrestrial and marine environments. Biotoxins, bacteria, viruses, helminths, acanthocephalans and protozoa represent a constant challenge to pinniped health and especially in pups with an immature immune system.

IV. 1. Nature of mortality factors and individual susceptibility

IV.1.1. Natural causes of mortality and epidemics

Causes of death are commonly categorized as “natural” and “non-natural”. Natural causes include all infectious diseases, neoplasia and parasitic infections whereas non-natural or artificial causes are in general referred to as the consequences of phenomena exterior to the normal biology of an individual or to the normal ecology of a species.

Studies investigating causes of death in pinnipeds are scarce and there are very few reports for otariid neonates in particular. The most frequently reported causes of pup mortality in pinnipeds include stillbirth, starvation, trauma, hookworm and bacterial infections (Lucas, 1899; Keyes, 1965; Mattlin, 1978; Anderson et al., 1979; Baker et al., 1980; Shaughnessy and Goldsworthy, 1990; Georges and Guinet, 2000; Soto et al., 2004). Causes of mortality in pups differ from those reported in older animals of the same species. These older animals may suffer from chronic infectious diseases, which include parasitic pneumonia and pulmonary tuberculosis.

Beside the usual levels of mortality, epidemic events have led to mass mortality in some pinniped populations (Laws and Taylor, 1957; Vedros et al., 1982; Geraci et al., 1982; Jensen et al., 2002). Pups were generally the most susceptible age group to succumb to these pathogens. Viruses such as phocine distemper virus and influenza virus are the agents most frequently reported as causing mass mortalities in pinnipeds (Geraci et al., 1982; Duignan et al., 1995; Jensen et al., 2002). Epidemics caused by bacteria seem less frequent, as judged by the fewer number of reports. Recurrent outbreaks of leptospirosis have been noted in California sea lions (Vedros et al., 1971; Smith et al., 1977; Gulland et al., 1996, 1999) and epidemics presumed to be due to *Campylobacter* sp. resulted in mass mortality events in crabeater seals (*Lobodon carcinophagus*) (Laws and Taylor, 1957) and in NZSLs (Baker, 1999).

IV.1.2. Toxic algal bloom and environmental contaminants

Global warming of the marine environment and ENSOs have occasionally led to toxic algal blooms. For example, brevetoxicosis caused by *Gymnodynium breve* and production of domoic acid by *Pseudonizschtia australis* have been associated with mass strandings and neurological signs in all age groups of various pinniped species off the coasts of Florida and California (O'Shea et al., 1991; Gulland et al., 2002).

Pinnipeds are also very sensitive to environmental pollutants such as organochlorine pesticides. These have been associated with immunotoxicity, carcinogenicity, growth and developmental abnormalities of foetuses and reproduction impairment (Bleavins and Aulerich, 1983; DeSwart et al., 1994, 1995; Beckmen et al.,1999, 2003).

IV.1.3. Genetic diversity of pinniped species and disease susceptibility

Wild populations tend to have a wide diversity of genes related to their immune system. This is believed to enable them to cope with emerging infectious diseases (Yuhki and O'Brien, 1990). Inbreeding has been commonly observed in isolated groups of individuals or declining populations. This has been observed to encourage homozygosity of genes

belonging to the Major Histocompatibility Complex (MHC) (Bowen et al., 2004) and probably genes for other traits. As a consequence, inbreeding has been implicated to reduce the ability of individuals in populations to develop resistance to infectious diseases as a result of the reduction of the genetic diversity in small populations of otariids (Hoelzel et al., 1999; Acevedo-Whitehouse et al., 2003a; Lento et al., 2003; Bowen et al., 2004). However, correlation between clinical susceptibility of individuals, as assessed by their health condition or natural immune responses, and their genetic profile has never been clearly demonstrated. The only research investigating such a relationship was conducted in California sea lions: it appeared that pups homozygous for a particular gene were predisposed to die from anaemia when infected with hookworms (Acevedo-Whitehouse et al., 2006).

IV. 2. Viral infections

For the past two decades, advanced studies on viruses in marine mammals, including pinnipeds, have characterized viral agents causing mass mortalities. Morbilliviruses, herpesvirus and influenza virus have caused major epidemics and high mortality in phocids (Geraci et al., 1982; Duignan, 1994; Harder et al., 1996; Van Bressem et al., 1999; Jensen et al., 2002). In contrast, there have been no reports of diseases caused by these viruses in otariids, despite serological evidence of exposure to phocine herpesvirus (Type 1 and 2) in the northern fur seal and Steller's sea lion (*Eumetopias jubatus*) (Osterhaus et al., 1987; Zarnke et al., 1997), suggesting that herpesviruses circulate in the marine environment worldwide. Only a few studies have focused on age-related susceptibility to viral infections and it appeared that in harbour and grey seals, pups were more severely affected by phocid-herpesvirus Type-1 than juveniles and older animals (Harder et al., 1997; Martina et al., 2002). This does not contradict the finding that harbour seal pups were fully immunocompetent at birth (Ross et al., 1994), since the later study referred to humoral immunity associated with viral infections in general whereas protection against herpesviruses infection is largely mediated by cellular immunity (Schmid and Rouse,

1992). As there are no such studies for otariids infected with viruses, it can only be speculated that fur seal and sea lion pups are likely to be the most susceptible to viruses.

Only the following two viruses, seal pox and San Miguel sea lion viruses, have been reported to cause clinical disease but not death in otariid hosts (Kennedy-Stoskopf, 2001).

IV. 2. 1. Seal pox

Seal pox has been commonly observed in a wide range of pinniped species including otariids. There are several reports in pups and juveniles in rehabilitation centres and in captivity (Wilson and Poglayen-Neuwall, 1971; Wilson et al., 1972; Osterhaus et al., 1990). Poxviruses cause nodular hyperplastic proliferative skin lesions on the head and flippers (Wilson and Poglayen-Neuwall, 1971; Wilson et al., 1972; Kennedy-Stoskopf, 2001). Most animals make a full recovery, usually in four to six weeks, but clinical signs may last for several months (Gage, 2003).

IV. 2. 2. San Miguel sea lion virus

This calicivirus cannot be distinguished from the virus that causes vesicular exanthema in swine. Several serotypes of calicivirus have been documented in pinnipeds and the opaleye fish is believed to be a reservoir for infection in California sea lions (Smith and Boyt, 1990; Barlough et al., 1998). Clinical signs are generally benign and infection is characterized by mild self-limiting vesicular skin disease on the flippers (Smith et al., 1998). A serological study showed that California sea lion pups had neutralizing antibodies to one or more serotypes by four months of age (Kennedy-Stoskopf et al., 1986). Ongoing exchange of serotypes between terrestrial and marine reservoirs could facilitate the development of humoral immunity to the various serotypes in pups. Given the limited pathogenicity of caliciviruses in pinnipeds, intensive studies do not seem warranted for this virus at this stage.

IV. 3. Bacterial infections

Bacterial infections are common in pinnipeds, involving a wide range of opportunistic and potentially zoonotic micro-organisms such as *Brucella* spp., *Leptospira* spp. and *Mycobacterium* spp. (Smith et al., 1974b; Sweeney and Gilmartin, 1974; Stroud and Roffe, 1979; Baker and McCann, 1989; Johnson et al., 1998; Thornton et al., 1998; Higgins, 2000; Hernández-Castro et al., 2005). Despite the continued development of diagnostic techniques, it remains difficult to quantify the role of bacterial diseases causing mortalities in wild marine mammals. Susceptibility to bacterial infections could be influenced by the host species (Thornton et al., 1998). Age class and immune status are also likely to contribute; however, there are no studies investigating these two latter factors in particular. As for viral epidemics (*see* Section IV.2), neonates and older debilitated animals are more likely to die from bacterial infections.

IV. 3. 1. Leptospirosis

Leptospirosis is a widespread bacterial disease in the marine ecosystem and has been associated with several epidemics in wild California sea lions along the Oregon and California coasts (Vedros et al., 1971; Gulland et al., 1996; Acevedo-Whitehouse et al., 2003b). The aetiological agent was identified as *Leptospira interrogans*, with the main serovars being Pomona and Hardjo (Gulland et al., 1996; Godinez et al., 1999). Evidence indicates that *L. interrogans* serovar Pomona is endemic in the California sea lion, causing recurrent outbreaks every three to four years (Gulland et al., 1996). Commonly observed clinical signs of leptospirosis include depression, abdominal pain and fever. Reproductive failure induced by *Leptospira* sp. was demonstrated in California sea lions, with abortions and multiple haemorrhages observed in foetuses and also neonates (Smith et al., 1974a). Lesions are generally found in kidneys and liver, and spirochetes have been seen on silver-stained histological preparations of kidneys (Dunn et al., 2001; Gage, 2003).

IV. 3.2. Tuberculosis

Mycobacterial infections have been diagnosed in wild and captive pinniped species of the Southern Hemisphere, including the NZSL, the New Zealand fur seal, the Australian fur seal (*Arctocephalus pusillus pusillus*) and the Australian sea lion (*Neophoca cinerea*) (Forshaw and Phelps, 1991; Cousins et al., 1993, 2003; Woods et al., 1995; Roe et al., 2006). Characterization of isolates of *Mycobacterium* spp. from pinnipeds of the Southern Hemisphere by spoligotyping (a technique of DNA fingerprinting, Kamerbeek et al., 1993) showed them to be a novel member of the *Mycobacterium* Tuberculosis Complex. The name *Mycobacterium pinnipedii* sp. nov. was proposed for this species found in Australian pinnipeds (Cousins et al., 2003). Whether the isolate from the NZSL (Roe et al., 2003) is the same species remains to be determined.

It seems that older animals are most likely to succumb to tuberculosis. However, mortality subsequent to mycobacterial infection was reported in captive juvenile New Zealand fur seals and in an Australian sea lion kept in the same facility, suggesting that younger animals are also susceptible (Forshaw and Phelps, 1991). The occurrence of tuberculosis in this case was more likely to be due to the introduction of infected wild animals rather than an indication of age differences in disease susceptibility per se. Although anorexia is the main presenting clinical sign, lung infection is almost invariably observed at necropsy, which suggests that pathogen transmission occurs via inhalation (Dunn et al., 2001). Mycobacterial infections of captive and wild animals represent a zoonotic risk (Forshaw and Phelps, 1991; Thompson et al., 1993).

IV. 3.3. Salmonellosis

Salmonellosis is primarily a gastrointestinal disease, characterized by lethargy, diarrhea and hemorrhagic enteritis in pinnipeds (Dunn et al., 2001; Gage, 2003). However, systemic salmonellosis has also been occasionally diagnosed (Howard et al., 1983). Infection with *Salmonella* spp. has been reported on at least one occasion to be a significant cause of pup mortality in the northern fur seal in Alaska (Jellison and Milner, 1958). Most reports of

salmonellosis in pinnipeds only refer to sporadic cases (Stroud and Roelke, 1980). *Salmonella* has been isolated from the faeces of clinically normal pups, e.g. northern fur seal and California sea lion pups on San Miguel Island in California (Gilmartin et al., 1979).

Various serotypes of *Salmonella enteriditis,* including Heidelberg, Cerro, Enteritidis, Newport and Typhimurium have been isolated from otariids worldwide (Cordes and O'Hara, 1979; Gilmartin et al., 1979; Vedros et al., 1982; Palmgren et al., 2000). Pathogenicity of *Salmonella* sp. serovars are known to vary and this may influence the occurrence of disease in otariids. In addition, it seems that serotypes isolated within the same population differ between years. This could be explained by the fact that pinniped migratory patterns lead them to inhabited coasts, where they can become infected with different *Salmonella* serotypes. Indeed, similarities between serotypes isolated in humans and wild animal species suggest that contamination of the marine environment by human waste is a possible source of infection for marine mammals (Smith et al., 2002; Fenwick et al., 2004).

IV. 3.4. Brucellosis

There is limited data available on the prevalence of clinical brucellosis and on the pathobiology of *Brucella* spp. infection in pinnnipeds. It has been associated with reproductive disorders in marine mammals (Dunn et al., 2001). There is strong serological evidence that brucellosis was geographically widespread in pinnipeds, with most reports in phocids (Foster et al., 1996; Nielsen et al., 2001; Garner et al., 1997; Jepson et al., 1997). Clinical disease due to *Brucella* spp. has not been diagnosed in marine mammals from Australia or New Zealand (Duignan, personal communication). However, there has been evidence of exposure of the New Zealand Hector's dolphins (*Cephalorhynchus hectori*) to this agent (Duignan, personal communication). Diagnosis of brucellosis in wild animals is not simple as culture conditions and media used for isolation of the bacteria strongly influence the outcome of the investigation (Miller et al., 1999). Serology is easier to apply as a diagnostic test but is not very specific or sensitive. More recently, immunoassay

techniques have been suggested to be a more reliable diagnostic method (Gall et al., 2000) but these have not yet been generally used in studies on pinnipeds.

IV. 3.5. Campylobacteriosis

Campylobacteriosis is a potential zoonotic disease and *Campylobacter* spp. have been isolated from stranded pinnipeds (Broman et al., 2000; Foster et al., 2004; Stoddard et al., 2005). However, in these animals, there was limited information to correlate the pathogen with clinical disease. *Campylobacter* spp. were incriminated as the cause of mass mortality in NZSLs in 1997/1998 on the Auckland Islands. These deaths were characterized by acute septicaemia causing necrotizing vasculitis and haemorrhagic pneumonia. Similar lesions were observed in a stranded New Zealand fur seal the following year (Duignan et al., 1999). Although preliminary genetic analyses suggested that the strain isolated in NZSLs could be related to *Campylobacter mucosalis* (Stratton and Duignan, unpublished), there was insufficient evidence to definitely show that this bacterial organism was causing this disease.

IV. 3.6. Miscellaneous bacterial diseases

A variety of other bacterial agents have been occasionally isolated from pinnipeds and implicated as possibly causing disease. Respiratory diseases predominate in post-mortem diagnoses of stranded pinnipeds. In general, bacterial pneumonia was caused by Gram negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella* spp. but *Staphylococcus aureus* was also frequently isolated (Sweeney and Gilmartin, 1974; Dunn et al., 2001; Gage, 2003). As expected, clinical signs included coughing, dyspnea, tachypnea and depression. There are limited reports of bacterial gastrointestinal diseases in pinnipeds apart from salmonellosis (see IV.3.3) and β-hemolytic *E. coli* (Diamond et al., 1980). Bacterial skin diseases are in most cases secondary to viral infections (for example seal pox virus); to trauma; or to external parasites (Dunn et al., 2001). Cutaneous abscesses are very common in pinnnipeds, and are usually directly associated with biting and fighting

during the breeding season (Baker and McCann, 1989). Bacteria isolated from these lesions include *Streptococcus* spp., *Staphylococcus* spp., *E. coli* and *Proteus* spp.

IV.4. *Klebsiella* spp. infections

IV. 4.1. Distribution and hosts

Klebsiella species are ubiquitous in both terrestrial and marine environments. In humans, *K. pneumoniae* has often been associated with high morbidity and mortality in hospitalized patients (Peña et al, 1998; Lebessi et al, 2002; Ben Hamouda et al, 2003). It is the second most frequent Gram-negative pathogen causing nosocomial septicaemia in humans (Hansen et al, 1999).

Klebsiella species have been commonly cultured from assorted internal tissues in marine mammals (Stroud and Roffe, 1979; Vedros et al., 1982; Baker and McCann, 1989; Hernández-Castro et al., 2005) and in a large range of wild birds, reptiles and terrestrial mammals (Enurah et al., 1988; Bartoszcze et al., 1990; Osawa et al., 1992; Aguirre et al., 1994; Dubay et al., 2000; Montgomery et al., 2002; Steele et al., 2005). Yet, *Klebsiella* species do not seem to have been reported to have caused mass mortality in a wild animal population.

IV. 4.2. Cross-reactivity between *Klebsiella* serotypes

Serotypes of *Klebsiella* species result from the variable combination of capsular polysaccharides (or K-antigens) and type-O lipopolysaccharides (or O-antigens) (Cortés et al., 2002). At present, 77 different K-antigens and at least nine O-antigens have been recognised (Hansen et al., 1999; Cortés et al., 2002). Such antigenic assortment has been applied in serology to identify serotypes for a *Klebsiella* species. However cross-reactivity between serotypes has been reported for a few decades (Cryz et al., 1986; Sechter et al., 2000). Classical serotyping techniques, including indirect immunofluorescent antibody (IFA) and Western Blot (WB) have described and the most commonly used will be

discussed in section V.1. The existence of cross-reactivity between K-antigens and of non-typeable *Klebsiella* strains (Fung et al., 2000) makes serotyping difficult to perform and interpret. However, a recent molecular serotyping method involving PCR-amplification of genes encoding for capsular antigens appears to be able to avoid cross-reactions encountered with non-specific serotyping techniques (Brisse et al., 2004).

IV. 4.3. Variations in pathogenicity

The pathogenicity of *Klebsiella pneumoniae* seems to depend on the degree of encapsulation of the bacteria. Indeed, both experimental studies and clinical observations have shown that only "heavily" encapsulated strains of *K. pneumoniae* were pathogenic (Jackson et al., 1980; Kikuchi et al., 1987; Postal et al., 1988; Lawlor et al., 2005).

IV.4.4. Genotyping *Klebsiella* species

Various genotyping methods have been used with *Klebsiella* spp. including pulsed-field gel electrophoresis (PFGE) (Hansen et al., 2002), amplified fragment polymorphism analyses (Jonas et al., 2004) and randomly amplified polymorphic DNA analysis (Wong et al., 1994). PFGE is the most widely used of these techniques (Gouby et al., 1994; Peña et al, 1998). These genotyping methods are now largely preferred to serotyping to discriminate *Klebsiella pneumoniae* strains during outbreaks. However, these methods are not considered appropriate for investigating the relatedness between geographically and temporally separate isolates. In this case, molecular serotyping techniques appear to be the most reliable option (Brisse et al., 2004).

IV.4.5. Lesions associated with *Klebsiella pneumoniae*

Post-mortem lesions induced by *K. pneumoniae* are very variable and can be found in a wide range of organs in numerous host species (Giles et al., 1974; Bonney et al., 1978; Jackson et al., 1980; Ling and Ruby, 1983; Timoney et al., 1983; Solleveld et al., 1984; Enurah et al., 1988; Coletti et al., 2001). *K. pneumoniae* was reported to cause haemorrhagic enteritis in rabbits (Coletti et al., 2001) and gastroenteritis and suppurative pneumonia in chinchillas (Bartoszcze et al., 1990). This pathogen was also isolated from

lesions of the respiratory system (Bonney et al., 1978; Enurah et al., 1988), urogenital tract (Ling and Ruby, 1983; Timoney et al., 1983) and laryngeal air sacs of animals (Giles et al., 1974). It is also known to be involved in septic arthritis in human neonates (Abuekteish et al 1996; Ryckewaert and Bardin 1996; Kao et al 2003), in cellulitis in an immunocompromised man (Park et al., 2004) and in liver abscesses of diabetic patients (Lau et al., 2000). However, because *Klebsiella* species are ubiquitous pathogens, these can be cultured from multiple organs, without necessarily causing disease.

IV.5. Parasitic diseases

IV. 5.1. Review of parasites found in pinnipeds

A vast range of parasites has been described in pinnipeds. In general, the life cycle of parasites infecting marine mammals are not very well documented. Most reports are from necropsy findings. Although parasitic diseases are often secondary to bacterial or viral diseases, some parasites may cause major heath problems in their pinniped hosts, especially when taking advantage of debilitated animals on the rookery.

Mites (including *Demodex* spp., *Sarcoptes* spp., *Orthohalarachne* and *Halarachne* spp.) and lice (e.g. *Anoplura* spp.) are ectoparasites of pinnipeds (see Table 1.1). The pathological role of these parasites is a function of their numbers but can consist of anaemia, pruritis and alopecia (Lauckner, 1985; Gage, 2003). Transmission of mites and lice generally occurs by close contact between animals (Gage, 2003).

Apicomplexans (*Eimeria* spp., *Sarcocystis* spp., *Toxoplasma gondii*) and flagellates (*Giardia* spp.) have been reported from phocids and otariids (Brown et al., 1974; Bishop, 1979; Migaki and Albert, 1980; Olson et al., 1997; Deng et al., 2000). Apicomplexans do not seem to play a considerable pathological role in parasitic infections of pinnipeds (Table 1.2) except for *T. gondii* (Van Pelt and Dietrich, 1973; Migaki et al., 1977) and *Eimeria phocae* in phocids (Hsu et al., 1974).

		Host / localisation	***Effect(s) on health***	***References***
Mites	*Demodex* spp.	phocids and otariids/ skin	no report of pathogenic effect	Lauckner (1985)
	Sarcoptes spp.			
Nasal mites	*Orthohalarachne* spp.	otariids / nares, nasopharynx, airways, lungs	sneezing, nasal discharge	Gage (2003)
	Halarachne spp.	phocids / nares, nasopharynx, airways, lungs		
Lice	*Anoplura* spp.	phocids and otariids/ skin	pruritis, alopecia, anemia	Gage (2003)

Table 1.1 Ectoparasites most frequently reported in pinnipeds.

Helminths include nematodes (“round worms”), trematodes (“flukes”) and cestodes (“tape worms”). In addition, pinnipeds are also infected with acanthocephalans (“thorny-headed worms”). There is a vast diversity of helminth species infecting pinnipeds and the combination of both morphological description and molecular analyses continue to refine the taxonomy in this area, especially with anisakids (Delyamure, 1955; Paggi et al., 1991; Mattiucci et al., 1997). Table 1.3 summarizes the most frequently reported helminths and acanthocephalans in pinnipeds. The only endoparasites occurring in pinniped pups are the nematodes *Uncinaria* spp., found in their small intestines.

		Host / localisation	*Effect(s) on health*	*References*
Apicomplexans	*Eimeria phocae*	adult phocids / large intestine	severe colitis	Hsu et al. (1974)
	Eimeria spp.	adult otariids / large intestine	no report of pathogenic effect	Drozda (1987)
	Sarcocystis spp.	adult phocids and otariids/ skeletal muscles	no report of pathogenic effect	Brown et al. (1974), Bishop et al. (1979), Migaki and Albert (1980)
	Toxoplasma gondii	phocids and otariids (adults and report in a 10 day-old captive pup)/ myocardium	myocarditis, necrotic lesions in liver, stomach, lymph nodes	Van Pelt and Dietrich (1973), Migaki et al. (1977)
Flagellates	*Giardia* sp.	adult phocids /intestines	unknown	Olson et al. (1997), Measures and Olson (1999), Deng et al. (2000)

Table 1.2 Protozoan parasites most frequently reported in pinnipeds.

			Host / localisation	*Effect(s) on health*	*References*
Gastrointestinal tract					
Nematodes	Ancylostomatidae	*Uncinaria lucasi, U. hamiltoni*	otariid pups (rare in phocid pups)/ small intestine	high pup mortality (haemorrhagic enteritis)	Olsen and Lyons (1965) Keyes (1965)
	Anisakidae	*Anisakis, Contracaecum, Pseudoterranova, Phocascaris* species	adult phocids and otariids/ stomach and intestines	gastric ulcers and nodules	Spraker et al. (2003)
Trematodes		*Galactosomum* sp., *Pricetrema* sp.	adult phocids and otariids/ intestines	no report of pathogenic effect	Delyamure (1955). Dailey and Brownell (1972)
Cestodes		*Diphyllobothrium* spp.	adult phocids and otariids/ intestines	occasionally obstruction of intestinal lumen in captive pinnipeds only	Lauckner (1985)
Acanthocephalans		*Corynosoma* spp.	adult phocids and otariids / large intestine	poorly documented	
Cardiorespiratory system					
Nematodes	Filaroididae	*Filaroides (Parafilaroides)* spp.	juvenile phocids and otariids/ lungs	pneumonia (common in stranded animals)	Lauckner (1985)
	Crenosomatidae	*Otostrongylus circumlitus*	juvenile phocids only/ major airways, pulmonary artery and heart	anorexia, depression, disseminated intravascular coagulation, death	Gulland et al. (1997)
	Filariidae	*Acanthocheilonema (=Dipetalonema) odendhali, A. spiraucoda, Dirofilaria immitis* (canine heartworm, captive pinnipeds only)	adult phocids and otariids / heart, lungs	anorexia, dyspnea, coughing	Gage (2003)
Liver and biliary ducts					
Trematodes		*Zalophotrema hepaticum*	adult phocids and otariids / liver, biliary ducts	moderate, thickened biliary ducts and possible obstruction in old animals	Gage (2003)
Connective tissue					
Nematodes		*Dipetalonema odendhali*	adult phocids and otariids/ intermuscular fascia	no report of pathogenic effect	Gage (2003)
Cestodes		plerocercoids of *Phyllobothrium* spp.	adult phocids and otariids/ blubber	no report of pathogenic effect	Dailey (1975)

Table 1.3 Helminths and acanthocephalans most frequently reported in pinnipeds.

IV. 5.2. Lungworm pneumonia

It is common to find lungworms in adult pinnipeds at necropsy (Sweeney, 1974; Gerber et al., 1993) but juveniles are particularly sensitive to parasitic pneumonia (Sweeney, 1974; Dailey, 2001; Gage, 2003). It has been suggested that this could be linked to the nutritional stress experienced by weanlings. At this age, they switch from a milk diet to live prey that are intermediate hosts to a diverse range of parasites, including *Filaroides* spp. and *Otostrongylus* spp. These parasites have an indirect life cycle and fish harbour infective larvae in their abdominal muscles or cavity (Anderson, 2000). The success of these first foraging trips is very variable and some lungworms may become pathogenic in malnourished animals (Gerber et al., 1993). Clinical signs of lungworm disease resemble those described in bacterial pneumonia (see IV.3.6). *Otostrongylus circumlitus* has been reported to be highly pathogenic in stranded yearling and juvenile northern elephant seals (Gulland et al., 1997).

IV. 5.3. Gastric nematodes causing ulceration

Similarly to lungworms, large roundworm infestations are a very common finding in pinniped stomachs at necropsy (Sweeney, 1974; Dailey, 2001; Gage, 2003). Both larval and adult stages can be found at the same time, suggesting that there is an ongoing infestation from the ingestion of intermediate hosts. Gastric nematodes, including *Contracaecum* spp., have been associated with the presence of healing ulcers and parasitic nodules in stomachs of subadult northern fur seals (Spraker et al., 2003). However, more severe lesions of ulceration have been frequently noted in debilitated and malnourished individuals. Other signs in these animals included dehydration, anaemia, and more rarely gastric perforation by nematodes, leading to peritonitis (Ridgway et al., 1975; Lauckner, 1985; Spraker et al., 2003).

IV. 5.4. Hemorrhagic parasitic enteritis

Pinniped pups (mainly otariids and occasionally phocids) are infected with the hookworm *Uncinaria* spp. Lesions associated with hookworm disease are very variable but usually include hemorrhagic enteritis and anaemia (Keyes, 1965; Brown et al., 1974; Lyons, 1963).

IV. 6. Hookworm infection in pinniped pups

IV. 6.1. *Uncinaria* species and their pinniped hosts

The hookworms *Ancylostoma* spp., *Necator* spp., *Bunostomum* spp. and *Uncinaria* spp. infect a vast range of mammalian hosts, including humans (Anderson, 2000). They belong to the family Ancylostomatidae. The only mode of transmission documented so far is the transmammary route (Olsen and Lyons, 1962). They measure up to two cm and have a direct life cycle. Adults anchor themselves deep into the small intestinal mucosa. As for *Bunostomum* spp., hookworms of the genus *Uncinaria* have a buccal capsule equipped with two cutting plates (Anderson, 2000). There is a sexual dimorphism and males are usually smaller than females.

The control of hookworms in otariid pups by anthelmintic drugs has been investigated on several occasions in northern fur seals (Lyons et al., 1978, 1980; Bigg and Lyons, 1981; Beekman, 1984). These studies aimed at finding a practical management tool to reduce hookworm-induced mortality in young pups, in the eventuality of the decline of otariid populations in Alaska (Lyons et al., 1978). Dichlorvos, disophenol and ivermectin were tested at variable concentrations and formulations. Dichlorvos seemed to be more effective than disophenol in removing adult hookworms from pups' intestines (Lyons et al., 1978, 1980; Bigg and Lyons, 1981). Transitory sign of toxicosis consisting of diarrhoea was observed in a few pups treated with dichlorvos (Lyons et al., 1980). The use of ivermectin in northern fur seals was reported in one study, concluding it was highly efficacious to

remove adult hookworms. However, there was no information on its effect on tissue larvae. Based on studies conducted in dogs and other domestic carnivores, ivermectin seems likely to be similarly effective against adult and larval stages of hookworms in otariid pups (Campbell, 1989).

Only two hookworm species, *Uncinaria lucasi* Stiles and *Uncinaria hamiltoni* Baylis, have been described from otariids (Stiles, 1901; Baylis, 1933, 1947). However, hookworms with intermediate morphotypes have been reported from pinniped hosts (Baylis, 1947; Olsen, 1952; Dailey and Hill, 1970; Nadler et al., 2000), suggesting that additional biodiversity may be present but as yet uncharacterized. In contrast, some authors have tended to confine the number of *Uncinaria* species infecting pinniped hosts to only the existing two, by naming subspecies of *U. hamiltoni* (Botto-Mañe and Garzon, 1975) or to a single one, *U. lucasi* (George-Nascimento et al., 1992). Taxonomy of *Uncinaria* species in pinnipeds requires further clarification and molecular analyses of the parasitic genome combined with morphometric descriptions of specimens should contribute to this enterprise (Nadler et al., 2000). Prior to the present research, there were no reports of hookworms in NZSLs and these parasites were reported only once in New Zealand fur seal pups in Australian waters (Beveridge, 2002).

Despite a single report of adult hookworms in the intestines of one Steller's sea lion (*Eumetopias jubatus*) sub-adult male (Olsen, 1958), all other reports have described the infection with adult parasites almost exclusively in otariid pups (Lucas, 1899; Lyons, 1963; Dailey, 1975).

IV. 6.2. Hookworm life cycle

The life cycle of *Uncinaria lucasi* has been fully described in northern fur seals by Lyons (1963) and Olsen and Lyons (1962, 1965) on the Pribilof Islands (Alaska). The transmammary route was then described as the mode of infection in fur seal pups, with infective larvae being ingested through the colostrum to nursing newborns. Maturation of larval stages to adult parasites then takes place in the small intestine of the pup. This is

similar to the development of *Ancylostoma caninum* in dogs following colostral intake (Burke and Roberson, 1985; Anderson, 2000). The cycle of *U. lucasi* was divided into three parts: (1) the free-living phase, with eggs developing into third-stage larvae (L_3) in the soil; (2) the tissue phase, with parasitic L_3 found in the hosts' tissues; and (3) the intestinal phase, with the ingestion of parasitic L_3 in the first milk and development into adult hookworms in the intestines of pups. It has not yet been established whether percutaneous infection can result in mature adult parasites in the intestines of any animal infected by this route. Indeed, although infective larvae have been found in the ventral and abdominal blubber of older pinnipeds on the rookery, including lactating females (Lyons, 1963), no adult hookworms were seen in their intestines. Presumably this is mostly likely to be because of some immunity acquired following neonatal infection with *Uncinaria* spp. The characteristic transmammary transmission discovered by Lyons (1963) in northern fur seals was also reported for *Uncinaria* spp in Juan Fernandez fur seals (Sepúlveda and Alcaíno, 1993) and in California sea lions (Lyons et al., 2000a). Several additional studies have compared measurements of larvae found in different tissues from animals of various ages (Olsen and Lyons, 1965; Lyons and Keyes, 1978; Sepúlveda and Alcaíno, 1993; Lyons et al., 2003). Larval sizes varied considerably depending on both the host's age and the tissue sampled, with larvae from the mammary glands of lactating females being longer than those found in the blubber of other adult fur seals. Recovery of *Uncinaria* spp larval stages from the soil revealed that free-living hookworm L_3 were ensheathed, were shorter than those found in the host's tissue (Olsen and Lyons, 1965; Lyons et al., 2000b) and sometimes could survive the winter temperature on the rookery in Alaska (Olsen, 1958).

IV. 6.3. Uncinariosis in pups

Hookworms have been associated with clinical disease in northern fur seal pups (Keyes, 1965; Kato, 1997; Lyons et al., 2001) and in California sea lion pups (Lyons et al., 2001). In contrast, infestation of Juan Fernandez fur seal and South American sea lion pups did not show any pathological changes at necropsy despite the presence of hookworms (Sepúlveda and Alcaino, 1993; Berón-Vera et al., 2004) although the burden reported in these two studies were relatively low.

In terrestrial mammalian hosts, hookworm disease is characterized by iron-deficiency anaemia, malabsorption and lethargy as reported in dogs and humans (Pitchumoni and Floch, 1969; Prociv, 1997; Hotez et al., 2004). Such pathological changes are the results of the feeding mechanisms of hookworms and the local damage and bleeding they cause in the intestines (Garside et al., 2000). In pinnipeds, lesions associated with hookworm disease are very variable but usually include hemorrhagic enteritis and anaemia (Keyes, 1965; Brown et al., 1974; Lyons, 1963).

In domestic carnivores, the young are more at risk of intestinal helminth infections such as hookworm disease, than older animals (Miller, 1965; Loukas and Prociv, 2001; Fujiwara et al., 2006). It seems that older animals have acquired a solid level of immunity to hookworms after repeated infections. Clinical expression of natural immunity to *Ancylostoma caninum* in dogs consists of reduced egg counts and lower numbers of adult hookworms in the intestines (McCoy, 1931; Kerr, 1936). There are no studies investigating immune response to hookworms in pinnipeds but it is likely that animals older than pups acquire such immunity after reinfection with *Uncinaria* spp. larvae on the rookery. It is not known how many times pinniped hosts need to be in contact with larval antigens to develop resistance to hookworm infection but in general, it seems that immunity to parasites in general (either larval stages of helminths or adult ectoparasites) takes longer to establish compared to immune responses to viral and bacterial infections (Banks, 1982; Morein et al., 2002). Chapters 3 to 6 are dedicated to studies involving hookworms in New Zealand sea lion pups.

IV.7. Mycotic diseases

There have been few reports on mycotic infections and their clinical manifestations in pinnipeds. In captivity, mycoses were diagnosed in apparently healthy adult animals and fungi were cultured from only a small percentage of neonates (Reidarson et al., 1999). Various species of pathogenic and non-pathogenic fungi have been recorded affecting a wide range of tissues, mainly the lungs and skin (Sweeney, 1974; Vedros et al., 1982; Migaki and Jones, 1983; Fauquier et al., 1996; Guillot et al., 1998). Clinical signs are non-

specific and pathogenicity of fungi is extremely variable. Biopsy and culture remain the most reliable methods to diagnose mycotic infections in pinnipeds (Reidarson et al., 1999).

V. Immunology in pinniped pups

V.1. Serological surveys in pinnipeds

Bacterial isolates in free-ranging pinnipeds include a wide variety of opportunistic and potentially zoonotic micro-organisms (*see* Section IV.3). Given the increasing risk of transmission of such pathogens to humans (Harvell et al., 1999) and reciprocally from terrestrial sources to marine animals (Grimes, 1991; Harvell et al., 1999; Stoddard et al., 2005), surveys have been conducted in wild and rehabilitated pinnipeds to monitor the prevalence of different bacteria and viruses present in the marine ecosystem and this is commonly undertaken using serological techniques.

Some care has to be taken in interpreting results when using serological techniques adapted from domestic animals to pinnipeds as only a few studies have characterized pinniped Igs (Cavagnolo and Vedros, 1978; Carter et al., 1990). They have shown that these were generally of a similar size and structure to other mammalian immunoglobulins. Hence it is reasonable to predict that most techniques used in other mammals could be adapted to use in pinnipeds.

When studying bacterial strains in wild pinniped populations, the Western Blot (WB) method presents several advantages compared to other serodiagnostic techniques. Antigens of infectious organisms are immobilized on a solid phase and the test serum probes these directly. The presence of pinniped antibodies aggregated on antigenic particles is revealed after incubation with a combination of enzyme/conjugate (staphylococcal protein A and/or streptococcal protein G) reagent and the corresponding substrate/chromogen reagent. Other techniques such as ELISAs require more effort to determine and validate cut-off values and may also depend on the degree of antigen purity (Aldridge et al., 2001; King et al., 2001).

Probably the main advantage in using WB is that it does not require a cut-off to be determined and results of the test are easier to interpret (Priest et al., 2001). However, WB remains more difficult to apply to a large number of samples and less sensitive than ELISA. There are also likely to be issues with cross-reactivity between serotypes (*see* Section IV.4.2). An alternative to serology is to search directly for the presence of organisms, its DNA, or its antigens.

V. 2. Passive transfer of immunity in pinnipeds

V.2.1. Placental *versus* colostral transfer

Passive immunity in mammals is transferred before birth through the placenta and post-partum through colostrum for a short period of time only. Pinnipeds have an endotheliochorial placenta like many carnivores including dogs (Dierauf et al., 1986; Carter and Enders, 2004). In such animals, transfer of Igs via the colostrum is expected to predominate over prenatal transfer, as endotheliochorial placentas are impermeable to Igs (Tizard, 1987). In a first study in southern elephant seals, Marquez et al. (1995) hypothesized that transfer of all IgGs occurred mainly before birth as none were detected in the milk throughout the whole lactation period. However, in a later study, after they had refined their technique, the authors then found out that all classes of Ig (G, M and A) were present in the milk but IgG was the predominant type from birth to the end of the lactation (Marquez et al., 2003). This suggests that transfer of Igs through colostrum is possible. Numerous studies support the evidence that colostrum is the major source of IgG in pinniped pups for the species so far studied (harbour seals: Ross et al., 1993, 1994; grey seals: Carter et al., 1990; northern fur seals: Cavagnolo and Vedros, 1979; northern elephant seals: King et al., 1998; southern elephant seals: Marquez et al., 2003). Nevertheless, the levels of Igs in serum of suckling pinniped pups (Carter et al., 1990; Ross et al., 1994; Beckmen et al., 2003) were generally lower compared to terrestrial species such as canids (10-20 mg/ml, Foale et al., 2003).

V.2.2. Absorption of maternal IgGs by the newborn

Absorption of Igs from the colostrum in pinnipeds presumably only occurs for a limited time as for other mammalian neonates (Casal et al., 1996). Intestinal closure is complete by 25 hours post-partum in bovine calves and feline kittens (Bush and Staley, 1979; Stott et al., 1979; Casal et al., 1996). After the phase of absorption, levels of maternal Igs may decline more or less rapidly, depending on the species.

V.2.3. Factors influencing the passive transfer of IgGs

There are various factors that can potentially influence passive transfer of Igs to pinniped neonates. Inexperience of the dam has been suggested to negatively affect transfer of immunity in pinnipeds. Indeed, northern fur seal pups born to young females were reported to have lower Ig levels compared to pups of older females (Beckmen et al., 2003). On the other hand, in a study with bovine species, it was reported that the levels of IgGs in calves' serum was lower in lactating females on diet restriction, even though there was no difference in IgG levels in the colostrum (Hough et al., 1990). This suggests that restricting the diet of pinniped lactating dams (*see* Section III.1) may influence the absorption of maternal Igs in the young. However, there are no studies investigating the influence of such a factor on passive immune transfer and on absorption of colostral Igs by pinniped neonates.

V.3. Immunocompetence of pinniped pups

Pups, like other mammalian neonates, benefit from maternal immunoglobulins for a limited period of time (Banks, 1982). Meanwhile they have to develop their own specific immune response to environmental pathogens. Infectious diseases endemic to a population tend to affect neonates when they are no longer protected by maternal Igs and while their immune system is still functionally immature (Morein et al., 2002). In addition, innate deficiencies in the immune system of mammalian neonates and other causes of impairment

of cellular immunity have been reported to diminish their immune response (Winter et al., 1983; Holan et al., 1991; DeSwart et al., 1994). As a consequence, maternally acquired Igs are crucial for pinniped pups to cope with their environment soon after birth; yet this protection is only temporary.

There are no techniques available to discriminate maternally-derived from innate Igs but it is possible to monitor the fluctuations of serum antibodies in neonates after experimental challenge of their immune system by vaccination against bacterial and viral pathogens (Spencer and Burroughs, 1992; Ross et al., 1994; Beckmen et al., 2003). Using this method, it was demonstrated that pinniped neonates are capable of developing a rapid, strong and highly specific humoral immune response (Ross et al., 1994; Beckmen et al., 2003). Harbour seal newborns even showed a stronger cellular immune response to rabies antigens than dog and cat neonates (Ross et al., 2004). Apart from studies using vaccination, there is no information on the ability of young pinnipeds to mount an immune response towards infectious antigens.

Conclusion of the literature review

The NZSL is an endangered species, endemic to New Zealand and breeding almost exclusively on the Auckland Islands. The population is declining and the occurrence of three seasons of mass mortality (in 1997/1998, 2001/2002 and 2002/2003) may have seriously compromised their chances of recovery.

All pinniped neonates depend on their dams to survive until weaning, which occurs later in otariids than in phocids. In fur seals and sea lions, lactating females alternate between short foraging trips and nursing periods ashore. Pups left unattended become more at risk of starvation and trauma. In the meantime, success of feeding at sea may be influenced by climatic fluctuations known as El Niño Southern Oscillations.

Stillbirth, starvation, trauma and bacterial infections are the most frequent natural causes of death reported in pinniped neonates. Hookworms are also noted to play a role in neonatal mortality of otariids. In addition, viral epidemics have caused high mortality in all age classes of some phocid populations. Anthropogenic activities, especially the fishing industry, have become factors of growing importance in pinniped mortality.

In their first weeks of life, pups are exposed to a broad range of infectious, climatic and environmental risks. Although passive transfer of immunity from the dam through colostrum seems less important in pinnipeds than in terrestrial mammals, pups appear to be relatively protected, presumably because they are capable of developing their own immune response to pathogens.

Research Objectives

Neonatal mortality has never been investigated in the New Zealand sea lion (NZSL) *Phocarctos hookeri*. Some causes of death such as stillbirth and trauma, commonly reported in other otariid species, have been observed at Sandy Bay Beach on Enderby Island (Auckland Islands). Yet neither prevalence, nor the importance of these factors has been established in NZSL pups.

The main directions of the present research were to identify causes of neonatal mortality in NZSLs in the Sandy Bay Beach sub-population, to assess their importance and to describe the lesions associated with them. This research used data collected during seven consecutive breeding seasons, 1998/1999 to 2004/2005. A vast panel of techniques were used to conduct this project, from histology to parasitology and from scanning electron microscopy to gross examination of pup carcasses.

Chapter 2

A survey of neonatal mortality in New Zealand sea lions (*Phocarctos hookeri*) at Sandy Bay on Enderby Island, Auckland Islands from 1998 to 2005.

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ABSTRACT– *Chapter 2*

As part of a health survey of New Zealand sea lions (*Phocarctos hookeri*) on Enderby Island, Auckland Islands (50° 30'S, 166° 17'E), neonatal mortality was closely monitored at the Sandy Bay colony for seven consecutive years. Throughout the breeding seasons 1998/99 to 2004/2005, more than four hundred post mortem examinations were performed on pups found dead at this site. The primary cause of death was categorized as trauma (35%), bacterial infection (24%), hookworm infection (13%), starvation (13%) and stillbirth (4%). However, diagnoses were not mutually exclusive and for most pups, more than one diagnosis was recorded. Every year, two distinct peaks of trauma were observed: the first associated with mature bulls fighting within the harem and the second with sub-adult males harassing pups. In the 2001/2002 and 2002/2003 epidemics caused by *Klebsiella pneumoniae,* mortality increased by three times the mean in non-epidemic years (10.2%). The increased mortality was attributed directly to acute suppurative infection attributed to the bacterium and also to an increase in traumatic deaths of debilitated pups. Parasitic infection with the hookworm *Uncinaria* spp. was a common finding in all pups older than three weeks of age and debilitation by the parasite may have contributed to increased susceptibility to other pathogens such as *Klebsiella* sp. or *Salmonella* sp. This study provides valuable quantitative data on the natural causes of neonatal mortality in New Zealand sea lions that could be used in demographic models for management of threatened species.

INTRODUCTION – *Chapter 2*

The New Zealand sea lion (NZSL), *Phocarctos hookeri* (formerly known as Hooker's sea lion) is one of the rarest and most locally endemic members of the Otariid family. Because of its restricted breeding distribution and limited population growth, the species was declared threatened in 1997 under provisions in the New Zealand Marine Mammals Protection Act 1978 (Molloy and Davies, 1994) and was listed as vulnerable by the IUCN (Reijnders et al., 1993). NZSLs breed on New Zealand's sub-Antarctic islands between latitudes 48°S and 53°S (Gales and Mattlin, 1997). Their population size is estimated at between 10,000 and 12,000 animals, comprising approximately 5,000 animals of mature age (Chilvers, unpublished data). This is one of the smallest population sizes reported for an Otariid species and available data suggests that this population is declining despite being protected since the late 1890s (Taylor, 1971; Wilkinson et al., 2003). Ninety percent of all breeding is concentrated on Dundas and Enderby Islands within the Auckland Islands group.

NZSL's highly localized distribution is a factor in their vulnerability to anthropogenic threats such as fishing on the Aucklands Shelf where between 17 and 140 sea lions are accidentally caught each season (Baird, 1996; Wilkinson et al., 2003). The full impact of fisheries on the sea lion population is unknown but several models suggest that this level of take may limit the species capacity to increase in number and under some scenarios result in population decline (Doonan and Cawthorn, 1984; Woodley and Lavigne, 1993). This uncertainty about anthropogenic impacts on the population is amplified when considering other natural regulators, such as disease, on population growth. Between 1997/1998 and 2004/2005 three epidemics (1997/1998, 2001/2002 and 2002/2003) occurred among NZSLs on the Auckland Islands (Baker, 1999; Wilkinson et al., 2006). The January 1998 event resulted in the death of 53% of that season's pups as well as an unknown number of adult animals (Baker, 1999). That event was the first evidence that disease plays a role in the demography of the NZSL. It also highlighted the lack of base-line health data on the species and provided the impetus for a directed survey of neonatal mortality at the Sandy Bay colony, the second largest and most intensively studied breeding site for NZSL. This paper reports the gross causes of mortality from 1998/1999 to 2004/2005 and discusses their importance and temporal variations within and between the breeding seasons.

Over the survey period from 1998/1999 to 2004/2005 daily counts of live and dead pups were undertaken at the Sandy Bay rookery on Enderby Island, Auckland Islands (50° 30'S, 166° 17'E) and accurate pup production estimates were conducted using direct counts and mark-recapture techniques (Gales and Fletcher, 1999; Wilkinson et al., 2003; Chilvers et al., 2006a). Pups were born between mid-December and mid-January with a mean parturition date at about the 26th of December (Cawthorn et al., 1985; Chilvers et al., 2006a).

For every breeding season from 1998/99, pups on the beach were observed at least twice daily from early December through to the middle of February. Thus, all carcasses were retrieved within at most, 12 hours of death. Carcasses from which all or most of the internal organs had been scavenged were not included in the study unless the cause of death was apparent. The animals were sexed, weighed (to nearest 100g), measured (length and girth) and examined externally for wounds, scars and ectoparasites before being necropsied using a standard post-mortem procedure (Duignan et al., 2003). The body condition was determined based on ventral abdominal blubber depth and liver weight. In fresh specimens whole blood was collected from the heart and centrifuged to harvest serum. A sample of blubber was stored in liquid nitrogen for fatty acid analysis as part of associated diet and foraging studies on this species. Both normal and abnormal tissues were fixed in 10% buffered formalin to be processed later at Massey University (Palmerston North, New Zealand) using routine procedures and stains for veterinary histopathology (Luna, 1993).

Tissues and organs routinely sampled for histopathology included skin, skeletal muscle, brain, spinal cord, tongue, tonsil, thyroid, trachea, lungs, heart, lymph nodes, diaphragm, liver, spleen, pancreas, adrenal glands, kidney, stomach, intestine, urinary bladder, and gonads. Samples for bacteriology were systematically collected from liver, lung, tonsil, thymus, lymph nodes, spleen, feces and any lesions or exudates and were stored in liquid nitrogen. Sheep Blood Agar, MacConkey and XLD (Xylose-Lysine Desoxycholate) agar plates were used for bacterial culture of selected samples: priority was given to internal lymphoid organs and lesions from pups diagnosed with bacterial

infection. Beside routine enzymatic tests (oxidase, catalase and coagulase), Microbact™ (MedVet Science Pty. Ltd, Adelaide, Australia), API®20NE (BioMérieux, France) and Rapid ID32 STREP® (BioMérieux, France) test kits were used to identify the micro-organisms. *Salmonella* isolates were sent to Environmental Science & Research (Porirua, Wellington, New Zealand) for phage-typing.

A sample of internal organs from 30 pups was selected for virology. Spleen was the organ of choice if available. However, in two animals abdominal lymph node was selected by default. The samples were homogeneized and co-cultured on Vero cells and incubated (37ºC, 5% CO_2 incubator) for 2 passages (seven days for each passage) and observed for cytopathic effects characteristic for morbilliviruses and herpesviruses.

The entire intestine was fixed in 10% formalin for later recovery of endoparasites. All recovered parasites (ecto- and endoparasites) were stored in vials containing 90% ethanol.

Cause of death was categorized as follows: stillbirth, trauma, malnutrition, infection with the hookworm *Uncinaria* spp. (Castinel et al., 2006), bacterial infections, and congenital defects inconsistent with life. Stillbirths were identified by pulmonary atelectasis, an attached fresh umbilicus, and often some autolysis of the carcass. Trauma included death from bite wounds, drowning or crushing causing regurgitation and aspiration. Malnourished pups were often below average birth weight (10.6 kg for males and 9.7 kg for females, Chilvers et al., 2006a), had no blubber layer and hepatic atrophy. Hookworm enteritis was characterized by copious bloody content in the intestine, serosal ecchymoses, high worm burdens, and anaemic carcasses. Bacterial infection included any or all of the following: septicaemia, suppurative pleuritis, peritonitis or meningitis, abscesses, suppurative or necrotizing pneumonia, and suppurative arthritis. Diagnoses were not mutually exclusive and any pup may have had more than one diagnosis ranked in order of significance as the cause of death. Diagnosis was regarded as open where no clear cause of death could be determined at post mortem examination.

Statistical analysis of data was undertaken using the statistical computer package SAS (Statistical Analyses System, Version 9.1; SAS Institute Inc., Cary, NC, USA). Neonatal mortality was analyzed using the GENMOD procedure with a logit model that considered the fixed effects of year, sex and their interaction. Likewise, each cause of mortality was analyzed using the GENMOD procedure with a logit model including the fixed effects of year, sex, and their interactions. Results were significant for $P \leq 0.05$. Prevalences are given as mean percentages.

In total, 455 pups were included in the survey which comprised 13.3% of the pups born at Sandy Bay colony and 92.3% of the total reported dead for this site over the study period (Table 2.1). For the entire period of study, the annual pup mortality (number of dead pups from early December to mid-February) on Sandy Bay beach ranged between 6.4% and 31.3%, with a mean of 14.4 % (Table 2.1). However, for the 2001/2002 and 2002/2003 breeding seasons, the mean mortality to mid-February (respectively 31.3% and 22.1%) was significantly higher compared to the other years (10.2%; $P<0.0001$). For the 2004/2005 season, the mortality rate returned to almost the same level as in the pre-*Klebsiella* epidemic seasons (Table 2.1). Where gender was reported, the number of necropsied males was not significantly different from the females over the seasons.

Season	Total born	Total dead	Mortality rate to mid-February (%)	Post-mortem*
1998/99	513	33	6.4	26
1999/00	506	44	8.7	34
2000/01	562	61	10.9	48
2001/02	403	126	31.3	126
2002/03	489	108	22.1	108
2003/04	507	67	13.2	62
2004/05	441	54	12.2	51
overall	**3,421**	**493**	**14.4%**	**455**

* does not include scavenged pups.

Table 2.1 NZSL pups born, found dead, and necropsied on Sandy Bay beach, Enderby Island, 1998/1999 -2004/2005. "Total born" corresponds to the mark-recapture estimates at 16th January. "Total dead" include data from early December to the 16th of February for each breeding season.

In non-epidemic years, 32.2 % of pups died from trauma, 17.0 % from starvation and 16.4 % from hookworm infection. Bacterial infections were responsible for 13.5 % of pup mortality outside the *K. pneumoniae* epidemics (Fig. 2.1). During these

epidemics, the proportion of death directly attributed to bacterial infections increased to 36.3%, which was significantly greater than in the non-epidemic years (P<0.0001). In general, bacterial infections were more prevalent following the epidemics (Fig. 2.1); however, the proportion as a primary cause of death returned to the same level as before the mass mortality events. The proportion of pups dead by trauma was not affected by the epidemics but the absolute number that died from trauma was increased in both of the epidemic years (Fig. 2.1). There were significantly fewer pups dying from starvation during and after the epidemics than before (P<0.005). Likewise, fewer pups died from hookworm infection during the 2001/2002 and 2002/2003 seasons, however, this trend was not significantly differentt (Table 2.2). Finally, the percentage of stillbirths was consistently low each year regardless of epidemics (4.2 %, Table 2.2).

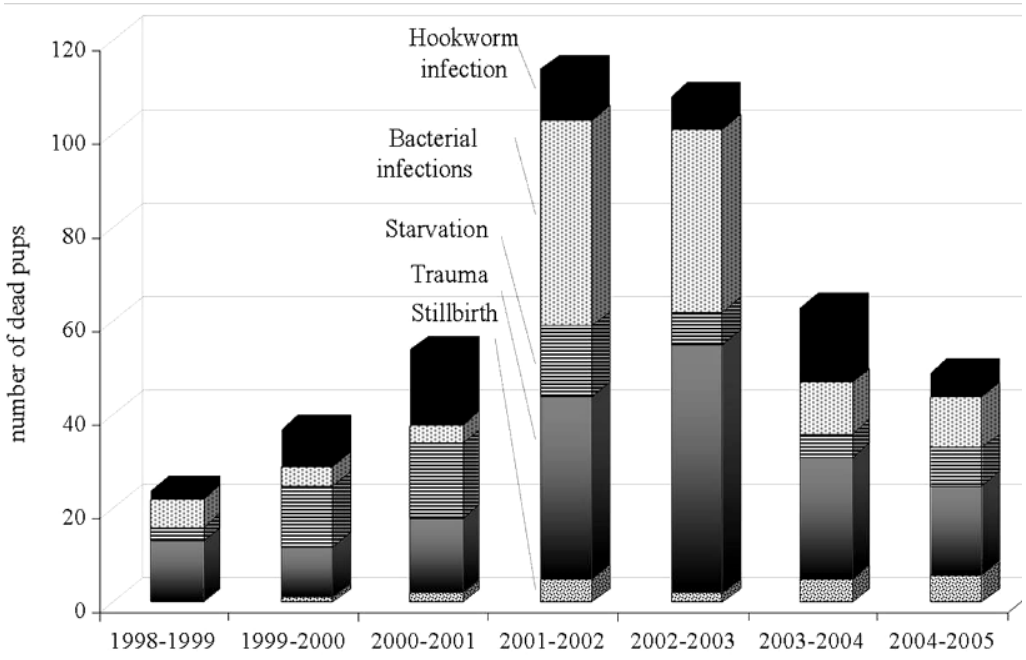

Figure 2.1 Prevalence of primary causes of mortality diagnosed in NZSL pups at necropsy at Sandy Bay Beach rookery, from 1998/1999 to 2004/2005.

	No necropsies	No. pups with diagnosis	Still birth	Trauma	Starvation	Bacterial infection	Hookworm infection
1998-1999	20	10	0	0	1[a]	5	2
1999-2000	34	34	2	7	13[a]	5	7
2000-2001	48	48	3	17	12[a]	3	13
2001-2002	128	120	3	15	15	46[b]	11
2002-2003	108	102	3	45	8	36[b]	10
2003-2004	62	52	5	25	6	9	17
2004-2005	51	50	6	17	8	12	7

[a] Significantly different from the following years ($P < 0.005$)

[b] Significantly different from the other years ($P < 0.001$)

Table 2.2 Distribution of the primary causes of neonatal mortality in New Zealand sea lion pups on Enderby Island, Auckland Islands for the 1998/1999 to 2004/2005 breeding seasons.

Temporal variations of causes of mortality for the years 2000/2001, 2001/2002, 2002/2003 and 2003/2004 are shown respectively in Fig. 2.2a, 2.2b, 2.2c and 2.2d representing one year before the *K. pneumoniae* epidemics, the two seasons of epidemics and the year immediately after that. Traumatic injuries as a primary cause of mortality had a consistent temporal trend each year from 1999/2000 to 2004/2005 regardless of epidemics. The first peak was observed during the month following the peak of parturition and coincided with dominant males fighting within the harem. A lesser, but still remarkable, increase of fatal traumatic injuries usually occurred later in February, this time associated with sub-adult and non-dominant males abducting pups.

The first diagnoses of hookworm infection at necropsy were made during the first week (in 2001/2002 and 2002/2003) or the second week of January (in 2000/2001 and 2003/2004), which is approximately three weeks after the annual peak of birth. This provides an insight on the pre-patent period of this parasitic disease that will be discussed later.

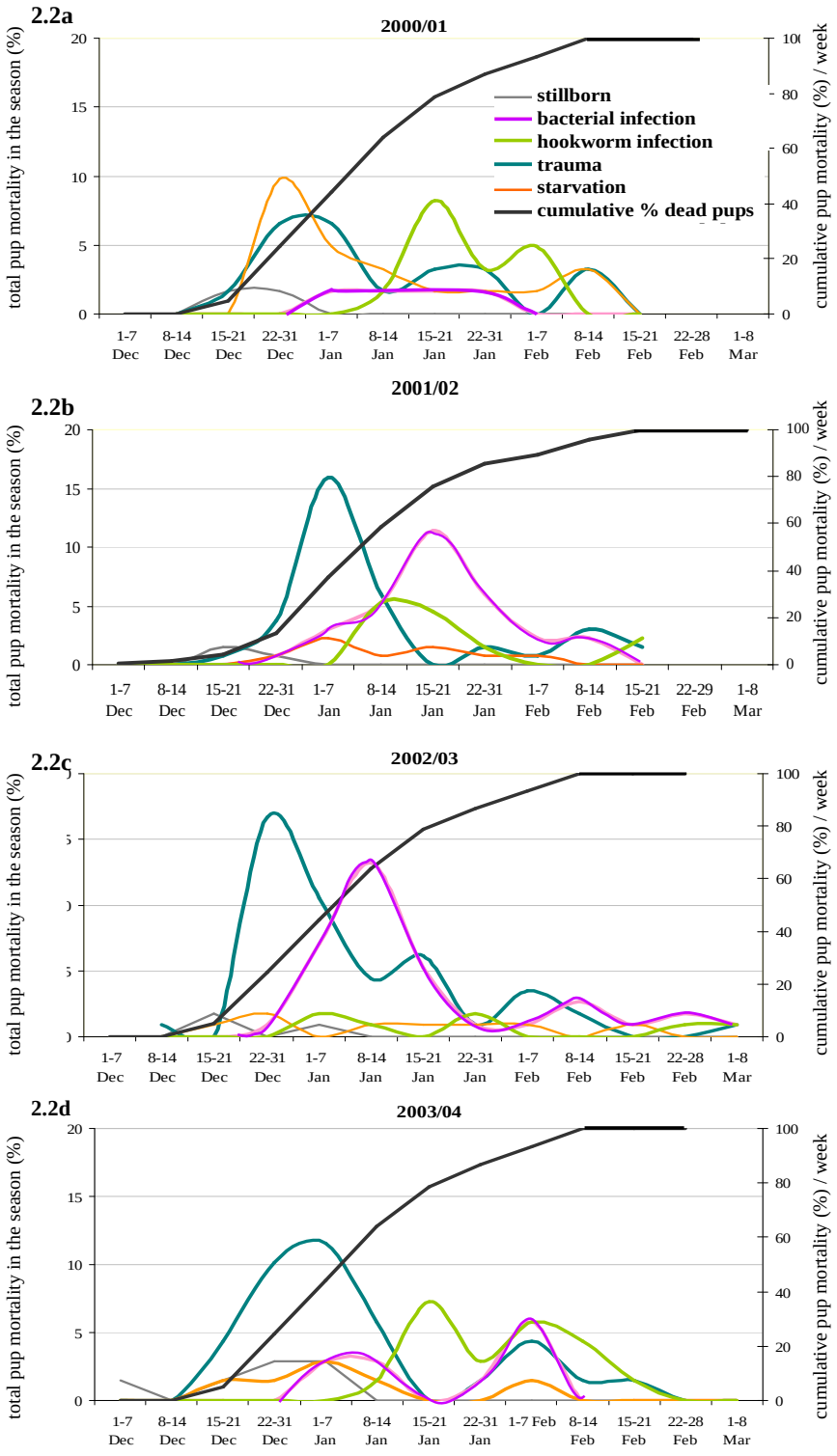

Figure 2.2 Incidence of primary causes of mortality in NZ sea lion pups on Enderby Island, Auckland Islands for the 2000/2001 (a), 2001/2002 (b), 2002/2003 (c) and 2003/2004 (d) breeding seasons.

In years prior to the epidemics, the most prevalent initial cause of death until mid-January was starvation; subsequently most pups died from hookworm infection until a rise in incidence of bacterial infections and trauma was observed in early February (Fig. 2.2a). The trends for the two epidemic years, 2001/2002 and 2002/2003 were comparable with the non-epidemic seasons for the incidence of all post-mortem diagnoses except that they differed by the marked increase in the number of pups killed by bacterial infections and trauma (Fig. 2.1, 2.2b, 2.2c).

Isolates from bacterial cultures included more commensal than pathogenic species (Table 2.3). *K. pneumoniae* was the most significant pathogen isolated from a wide range of tissues and lesions in both 2001/2002 and 2002/2003 and it was also isolated from some dead pups in the years subsequent to the epidemics. However, attempts to isolate this pathogen from archived samples collected prior to the 2001/2002 epidemic failed. Several different *Salmonella enteriditis* subspecies (serotypes Cerro, Derby and Newport) were also identified from pup feces collected at necropsy over the whole period of study. In 2001/2002, six of six cultured faecal samples were *Salmonella* positive, in 2002/2003, 17 of 19 samples (89.5%) were positive and in 2003/2004, *Salmonella* was cultured from 9 of 14 samples (64.3%).

Of the thirty virology cultures, two were contaminated with *Aspergillus* spp., making any interpretation impossible, and all the others were negative after two passages on Vero cells for viruses identified by cytopathic effect.

A low incidence of congenital anomalies was reported (12/476 or 2.6%). In some cases those defects, such as *atresia coli*, were not compatible with life while others were incidental findings in pups where another primary cause of death was identified (Table 2.4).

Tissue	Before epidemics	Epidemics	Post epidemics
Lymphoid tissues (tonsil, lymph nodes, spleen, thymus)	*Escherichia coli* (2/3); *Streptococcus sp* (1/3); *Salmonella enteriditis* subspp (1/3)	*Klebsiella pneumoniae* (41/45); *Staphylococcus intermedius* (1/10); *Staphylococcus aureus* (1/17); *E. coli* (3/25); *Streptococcus canis* (1/2); *Streptococcus dysgalactiae* subsp. *Equisimilis* (2/12)	*K.pneumoniae* (33/70); *Klebsiella oxytoca* (5/66); *E. coli* (7/42); *Salmonella enteriditis* subspp (1/25); *Streptococcus* spp (3/57); *Staphylococcus* spp (6/38); *Proteus* spp (4/25)
Subcutaneous tissues (incl. muscles, abscesses)	*Streptococcus* spp (1/1)	*K.pneumoniae* (11/12); *Proteus* spp (1/6); *Streptococcus* spp (1/6); *E. coli* (1/6)	*Staphylococcus spp (1/1) K.pneumoniae (2/9); E.coli (1/9); Staphylococcus spp (1/9)*
Joints fore-flipper (shoulder, elbow, metacarpus, carpus)	-	*K.pneumoniae* (32/34); *Staphylococcus intermedius* (1/13); *E.coli* (2/18); *Streptococcus canis* (1/14)	*K.pneumoniae* (18/32); *K.oxytoca* (4/28); *Streptococcus spp* (4/24); *Staphylococcus* spp (1/8); *E.coli* (1/20); *Shigella* spp (1/4)
Joints back-flipper (hip, metatarsus, tarsus)	-	*K.pneumoniae* (8/11); *Proteus* spp (2/4); *Staphylococcus intermedius* (2/4); *E. coli* (1/7)	*K.pneumoniae* (1/3); *Staphylococcus* spp (1/3)
Liver, Pancreas	*K. oxytoca* (1/3); *Staphylococcus* sp (1/3); *E.coli* (1/3)	*K.pneumoniae* (3/3)	*K.pneumoniae* (2/2)
Lung	*Salmonella enteriditis* subsp (1/4); *Staphylococcus aureus* (1/4); Proteus sp (1/4); *Streptococcus* sp (1/4)	*K.pneumoniae* (3/4); *E. coli* (1/4); *Streptococcus canis* (1/4); *Proteus* sp (1/4)	*K. pneumoniae* (7/20); *E.coli* (5/20); *Staphylococcus* sp (1/20); *Streptococcus spp* (3/20)
Abdominal cavity (peritoneum)	*E. coli* (1/2); *Streptococcus* sp (1/2)	*K. pneumoniae* (6/6)	*K. pneumoniae* (6/26); *K.oxytoca* (3/9); *Salmonella enteriditis* subsp (1/9)
Spino-cerebral tissue (meninges/brain)	*Staphylococcus* sp (1/2); *Streptococcus* sp (1/2)	*K. pneumoniae* (6/8); *Staphylococcus intermedius* (2/8); *Proteus* spp (2/8)	*K. pneumoniae* (5/8); *E. coli* (1/8); *Streptococcus* sp (1/18)
Thoracic cavity		*K. pneumoniae* (2/3)	*K.pneumoniae* (1/4); *K.oxytoca* (1/4); *E. coli* (1/4)
Kidney	*E. coli* (1/1)	-	-
Pericardial fluid/Myocardium	-	-	*K.pneumoniae* (2/4); *Staphylococcus* spp (2/4)

Table 2.3 List of bacteria isolated from various tissues of New Zealand sea lion pups, 1998/1999 to 2004/2005 comparing ratio in years of epidemic caused by *Klebsiella pneumoniae* (2001/2002 and 2002/2003) to years before (1998/1999, 1999/2000 and 2000/2001) and after epidemics (2003/2004 and 2004/2005).

Congenital defect	Number affected	Cause of death
Intestinal atresia	4/12 (33%)	Atresia coli
Ventricular septal defect	1/12 (8%)	Cardiac anomaly and malnutrition
Hypoplasia of one adrenal and one thyroid gland	1/12 (8%)	Hookworm enteritis and malnutrition
Right renal aplasia and polycystic left kidney	1/12 (8%)	Acute suppurative pleuritis and vaginitis
Right thyroid hypoplasia and left thyroid hyperplasia	1/12 (8%)	Malnutrition
Hiatus hernia	1/12 (8%)	Hookworm enteritis.
Scoliosis of cervical spine	1/12 (8%)	Malnutrition.
Multiple spinal, cranial and visceral anomalies.	2/12 (17%)	Multiple congenital defects inconsistent with life.

Table 2.4 Congenital defects in NZSL pups found dead at Sandy Bay Beach 1998/1999 to 2004/2005 with incidence and cause of death.

The seven continuous years of monitoring allowed the main causes of neonatal mortality to be identified. These included in order of prevalence: trauma, bacterial and hookworm infections, starvation and stillbirth. Furthermore, the data enabled an analysis of the impact of variables such as year, sex, age, and stochastic events (epidemics) that could influence the mortality rate and causes. There are limited studies on other pinniped species, and in particular on otariid species, with which to compare this survey. Most of the previously published reports present data for only a short period, usually for one breeding season (Mattlin, 1978; Baker et al., 1980; Georges and Guinet, 2000). Furthermore few other studies have had detailed demographic data over a long period on births and deaths and had access to almost all of the dead pups for detailed necropsy and sampling. As such, this is a relatively unique study.

Overall the mortality recorded for non-epidemic years in the present study for NZSL is not unusually high compared to other pinniped populations not subjected to unusual events. Thus, the mean mortality in NZSL neonates was 10.2% which is comparable to 12.5% in grey seals (*Halichoerus grypus*) from the Isle of May sampled in 1986 (Baker and Baker, 1988), 11.25% in California sea lions (*Zalophus californianus*) from Baja California between 1982 and 1985 (Aurioles and Sinsel, 1988), and 13% for South American sea lions (*Otaria byronia*) in years not affected by El Nino (Soto et al., 2004). Slightly higher (20%) mortality has been reported in the New Zealand fur seal, *Arctocephalus forsteri* (Mattlin, 1978). Stochastic events such as El Niño/La Niña cycles can have devastating effects on neonatal otariids, and cause up to 100% mortality in species such as the South American sea lion, the Galapagos fur seal (*Arctocephalus galapagoensis*) and the California sea lion (Francis and Heath, 1991; Trillmich and Dellinger, 1991; Soto et al., 2004).

The number of NZSL dead pups per week was higher in January when they were from 1 to 5 weeks-old with 50% of the seasonal pup mortality reached in the first week of January when pups were about two weeks of age. The only comparable data on pup mortality in relation to age were found in studies on fur seals. In Sub-Antarctic fur seals (*A. tropicalis*), the mortality rate was higher from birth to two weeks-old (9%) and

decreased to 3.2% between two weeks and one month of age (Georges and Guinet, 2000). In Antarctic fur seals (*A. gazella*) at South Georgia, half of neonatal mortality had occurred by the age of two days and 90% by one month (Doidge et al., 1984). These comparisons suggest that there is a similar trend between studies and that otariid neonates in general are more at risk in their first few weeks of life.

The ratio of males to females at necropsy in NZSL showed that there was no difference between sexes in pup mortality, a similar finding to that reported in Sub-Antarctic fur seals on Amsterdam Island (Georges and Guinet, 2000). This is important when considering population management and sex-specific survival rate. During the neonatal period, mortality does not seem to affect the sex ratio calculated at three months of age but events in the following years such as male aggression on females may upset this parity (Chilvers et al., 2005a).

In 1997/1998, a mass mortality event killed 60% of NZSL neonates and many adults at all three Auckland Islands rookeries (North Auckland Islands, Dundas and Figure of Eight Islands). It was not possible to attribute the mortality to a single causative agent because too few necropsies were carried out at that time (Baker, 1999). However, this event initiated the study reported here and facilitated contingency planning by the New Zealand Department of Conservation for future events (Baker, 1999). Thus when further epidemics occurred in 2001/2002 and 2002/2003, detailed sampling was conducted and the aetiology determined. Mortality associated with bacterial infections was increased threefold and the pathogen *K. pneumoniae* was isolated in pure culture from almost all necropsied pups (Wilkinson et al., 2006). *Klebsiella* species are widespread pathogens capable of causing severe nosocomial outbreaks in humans hospitalized in intensive care units and in neonatal wards resulting in septicaemia (Peña et al., 1998; Lebessi et al., 2002). *Klebsiella* species have been commonly cultured from various internal tissues in marine mammals (Stroud and Roffe, 1979; Vedros et al., 1982; Baker and McCann, 1989; Hernández-Castro et al., 2005) and in a vast range of wild birds, reptiles and terrestrial mammals (Enurah et al., 1988; Bartoszcze et al., 1990; Osawa et al., 1992; Aguirre et al., 1994; Dubay et al., 2000; Montgomery et al., 2002; Steele et al., 2005). Yet, *Klebsiella* species have never previously been reported to cause an epidemic in an animal population (Wilkinson et al., 2006). The origin of infection here is

unknown but the lack of isolates from samples collected prior to 2001/2002 suggests a recent introduction of the pathogen into this population. There was no evidence either that viral infection increases susceptibility of the sea lion pups to a commensal *Klebsiella* already in the population.

The present study ranked bacterial infections the third cause of direct mortality in NZSL neonates in non-epidemic years (after trauma and hookworm disease) but they were recorded as the major cause of death in grey seal pups in two studies (Anderson et al., 1979; Baker et al., 1980). In NZSLs, bacterial infections as a primary diagnosis accounted for less than 15% of pup death outside the *K. pneumoniae* epidemic years (Fig. 2.2). However, the overall prevalence remained intermediate in the years following the epidemics. Since *K. pneumoniae* was frequently cultured from pup tissues and was still causing septicaemia after 2002/2003, it is likely that the immunity against these bacteria was not complete or at least not successfully transferred to pups through the colostrum. Immunization of a naïve population towards an introduced pathogen may take several years before neonates receive sufficient maternal antibodies to overcome the critical first months of life (Carter et al., 1992). In addition to having an immature immune system, NZSL pups may be at risk of infection because of the sandy substrate on which they live. The abrasive action of sand and a dirty environment has been associated with navel infection that evolves into peritonitis and septicaemia after passage of the pathogens through the liver. This "peritonitis-navel ill complex" has been observed in other pinnipeds. It contributed for 50% of pups' death in grey seal colonies (Anderson et al., 1979; Baker and Baker, 1988). Somewhat surprisingly, although Sandy Bay rookery consists of a beach with small particles of sand and the density of pups can be high in some areas, there were only a few cases of omphalitis diagnosed during the survey and cases of peritonitis were not associated with the occurrence of omphalitis. Most of the bacteria involved in these cases of septicaemia were *Klebsiella pneumoniae* (during and after the *K. pneumoniae* epidemics), *Streptococcus* spp. and *Escherichia coli.*

The different categories of primary causes of death in NZSL pups, in epidemic and in non-epidemic years, were similar to those described in other pinniped species although their relative ranking in order of importance did vary. For instance, Keyes

(1965) reported that the primary diagnoses of neonatal mortality in the northern fur seal were by order of importance, malnutrition, trauma, hookworm infection and miscellaneous bacterial infections. In NZSLs, trauma was the predominant cause of pups' death in all years. Signs of suffocation as well as cranial and thoracic haemorrhage in pups less than two weeks old were the common presentations of trauma caused by adult animals. Later in the breeding season, internal abdominal haemorrhage, bites and skin lesions were correlated with sub-adult and peripheral males physically abducting pups while lactating females were feeding at sea (Chilvers et al., 2005a). Moreover, Wilkinson et al. (2000) suggested that pup abduction associated with infanticide and cannibalism in NZSLs could be a significant cause of neonatal mortality. However, in the present study and in other otariid species (Australian sea lions, *Neophoca cinerea*: Higgins and Tedman, 1990; northern fur seals: Kiyota and Okamura, 2005; Antarctic fur seals: Doidge et al., 1984), male harassment and abduction causing death of pups seem to be the result of misdirected aggression or an accidental occurrence. Neonates would simply be too small and too slow to avoid the attacks and to survive the injuries that are likely to be insignificant in older animals.

Topography and density of animals on the rookery may affect neonatal survival. For instance, the open Sandy Bay beach does not provide any shelter for NZSL pups to hide from adult males and scavenging birds; the same hazardous conditions were reported for Sub-Antarctic fur seals (Georges and Guinet, 2000) and grey seals (Twiss et al., 2003). In contrast, rookeries on rocky shores with boulders and crevices provide shelter for fur seal pups from fighting males (Bradshaw et al., 1999; Kiyota and Okamura, 2005). In addition, in Antarctic fur seals, there is evidence that frequency of aggressive interactions increases when colony density becomes high (Doidge et al., 1984; Reid and Forcada, 2005). Thus, it is possible that infanticide and cannibalism reported in NZSL on Dundas Island by Wilkinson et al. (2000) could be linked to the four-fold higher number of animals in that rookery compared to Sandy Bay (Enderby Island) where no such behaviour was observed during the present survey. Likewise, Baker and Baker (1988) noticed that the increase of grey seals concentration at high tide generated more conflicts within the colony, resulting in more frequent bites and trauma to pups.

Starvation is recognized as an important cause of death in pinniped pups. The estimate of 13% in this study is similar to that reported in grey seals by Anderson et al. (1979) but contrasts with other studies describing starvation as the major factor of neonatal mortality in New Zealand fur seals (Mattlin, 1978) and in Hawaiian monk seals, *Monachus schauinslandi* (Banish and Gilmartin, 1992).

It is most likely that starvation in NZSL neonates was primarily due to death of the mother. This may happen for various reasons such as shark predation, disease, or male aggression (Wilkinson et al., 2000; Chilvers et al., 2005a). Given that lactating NZSL females forage at great distances from the rookery and at greater depth than most other otariids it is thought that they are living at their physiological limits (Costa and Gales, 2000; Chilvers et al., 2006a). Thus, NZSLs would seem to be susceptible to fluctuations of food resources as caused by El Niño/La Niña events or other climatic fluctuations (Renwick, 2005). The impact of these events have not been investigated in NZSL but foraging patterns of other marine species sharing the same latitudes have been studied in parallel with El Niño/ La Niña events (Boyd et al., 1994; Peacock et al., 2000; Bowen et al., 2001; Soto et al., 2004). Availability of prey in the equatorial Pacific region varies considerably between El Niño (low productivity) and La Niña (high prey abundance) Southern Oscillations (Barber and Chavez, 1983; Glynn, 1988). Periods of low prey abundance have led the lactating females of some otariid species to extend the duration of their foraging trips with increased activity and energy expenditure (Cape fur seals, *A. pusillus pusillus*: Bowen et al., 2001; Antarctic fur seals: Boyd et al., 1994). When female Cape fur seals cannot cope with extreme physiological conditions, they abandon their pup (Bowen et al., 2001). Similarly, pup growth in NZ fur seals partly reflected efficiency of lactating females and food availability (Bradshaw et al., 2000). In the present study, there does not appear to be any relationship between the occurrence of El Niño/ La Niña years (1997/1998 for El Niño and 1998/1999 for La Niña) and starvation and malnutrition in NZSL pups. Although the first recorded NZSL epidemic was concurrent with 1997/1998 ENSO event any association between it and the epidemic was speculative (Baker, 1999).

Uncinaria spp represent a constant primary cause of pup mortality in this colony of NZSL. Every year, adult hookworms and associated intestinal lesions could be

observed for the first time about three weeks after the majority of pups were born at Sandy Bay rookery. Thus it would appear to take about three weeks for the infective larvae to develop into mature adults feeding on the intestinal mucosa in NZSL pups (Castinel, unpublished; *see* Chapter 6). This suggestion depends on the hypothesis that transmammary transmission is the exclusive mode of transmission in this species, as has been previously described in northern fur seals by Olsen and Lyons (1965). Although the prevalence of infection is high, only a minority of pups succumb to the parasitic disease as a primary cause of death. The lower proportion of pups diagnosed with hookworms in 2001/2002 and 2002/2003 is more likely to be the result of pups dying from acute bacterial infection and septicaemia before three weeks of age rather than a lower parasitic pressure during the epidemic years.

Hookworms and to some extent any parasites (bacteria, viruses, *sensu* May and Anderson, 1979) may play a role in pup mortality by causing debilitation and death from trauma, malnutrition and hypothermia. Such a scenario was also reported for Hawaiian monk seal neonates (Banish and Gilmartin, 1992) and for northern fur seal pups (Spraker et al., 2004). To further investigate this hypothesis in NZSLs, studies are under way to correlate infection by enteric bacteria with the extent of lesions caused by *Uncinaria* spp. embedded in the mucosa.

Viral infections have been associated with neonatal mortality in other pinniped species. In particular Herpes virus infection caused by phocid herpes viruses (PhHV-1 and PhHV-2) have been isolated from harbor seal (*Phoca vitulina*) neonates that died in a seal rehabilitation center from pneumonia or adrenal necrosis (Osterhaus et al., 1985; Gulland et al., 1997b). Morbillivirus infection (Phocine Distemper virus) may also cause neonatal mortality in phocid seals but has never been seen in otariids (Duignan et al., 1993; Duignan et al., 1995; Daoust et al., 1993). It was also suggested that caliciviruses may be involved in abortion and neonatal death in California sea lions (Smith et al., 1979) In the present study, no viral infections were identified in tissues cultured from NZSL pups but the limited number of samples tested could not definitively rule out infection in the population.

CONCLUSION – *Chapter 2*

This study is the first to report the main factors of mortality in pups from birth to three months of age in NZSLs. Stillbirth, starvation, and hookworm disease remained relatively consistent over the seven years of the survey while bacterial infections and trauma went through a dramatic increase for two years of epidemics caused by the bacteria *Klebsiella pneumoniae*. Even if the equilibrium between primary causes of mortality was proportionally reestablished soon after, long-term consequences of pup loss are yet to be determined in the demographics of this threatened species (Wilkinson et al., 2006). Aside from the demographic impact of stochastic events such as the *Klebsiella* epidemics, it is now possible to factor in more consistent causes of mortality such as hookworm and starvation into the demographic models for this species. Thus, the data presented here should assist in the management of this threatened pinniped species.

Chapter 3

First report and characterization of adult *Uncinaria* spp. in New Zealand Sea Lion (*Phocarctos hookeri*) pups from the Auckland Islands, New Zealand.

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ABSTRACT – *Chapter 3*

Two species of hookworms (*Uncinaria lucasi* and *Uncinaria hamiltoni*) have been formally described from pinnipeds but dissimilar types are noted from these hosts. This is the first description of hookworms (*Uncinaria* spp.) from the New Zealand sea lion (NZSL), *Phocarctos hookeri.* The nematodes were collected from dead pups on Enderby Island (Auckland Islands, 50°30'; 166°17') during January and February, 2004. Standard measurements of male and female hookworms were obtained, providing a general morphometric characterization of the hookworm species in *Phocarctos hookeri.* Considerable variations in the body length of adult hookworms were noted within the same host. The arrangement of some of the bursal rays differs from that described for *U. lucasi* and *U. hamiltoni*.

INTRODUCTION – *Chapter 3*

The New Zealand Sea Lion (NZSL), *Phocarctos hookeri* (Hooker's sea lion) is one of the rarest and most locally endemic members of the Otariidae. This species has been classified as "vulnerable" and "endangered" because of its small population size and limited distribution on the subantarctic Auckland Islands (Gales and Fletcher, 1999). Studies conducted during the past seven years on Enderby Island (Auckland Islands) have shown that hookworm infection is a significant cause of mortality in pups (Castinel et al., 2004).

Only two hookworm species, *Uncinaria lucasi* Stiles and *Uncinaria hamiltoni* Baylis, have been described from otariids (Stiles, 1901; Baylis, 1933, 1947). However, individual hookworms with intermediate morphotypes have been reported from pinniped hosts (Baylis, 1947; Olsen, 1952; Dailey and Hill, 1970; Nadler et al., 2000), suggesting that additional biodiversity may be present but as yet uncharacterized. The present study provides a morphometric description of hookworm specimens collected from infected NZSL pups on Enderby Island. This is necessary for more comprehensive comparisons of *Uncinaria* spp. from various pinniped hosts.

MATERIALS AND METHODS – *Chapter 3*

Necropsies to investigate neonatal mortality were conducted in the field on NZSL pups found dead on Sandy Bay beach, Enderby Island (Auckland Islands, 50°30'; 166°17') during the period from January 7th to February 14th 2004. Mature adult hookworms were collected from the intestinal tract of four to seven week-old pups and stored in 100% ethanol. Thirty mature specimens were selected from four different pups –which individual burden ranged from 2310 to 7080 parasites- for morphological study, cleared with a few drops of lactophenol and mounted temporarily on slides. The hookworms were examined using a light microscope and images acquired using a digital camera. Morphological study concentrated on features used previously to characterize *U. lucasi* and *U. hamiltoni* including the buccal capsule, oesophagus, male copulatory bursa, and female tail. Fertile eggs (eight cells-stage) were obtained by dissection of the uterus of females and measured. Oesophageal length was measured from the beginning of the oesophageal tube (at the base of the buccal capsule) to the junction with the intestine; this differs from the variable measured by Baylis (1933) and Olsen (1952). In addition, some specimens of both genders were prepared for Scanning Electron Microscopy (SEM) following standard procedures. Eight adult males and 11 adult females were measured using a microscope equipped with an ocular micrometer (Table 3.1).

Measurements (in text) are means followed by standard errors (ranges are provided in Table 3.1). Ratios of some features were used to compare measurements between males and females. Analyses consisted of descriptive statistics and one-way ANOVAs to investigate the effect of gender on the measured variables. Adult *Uncinaria* spp. specimens of each sex were deposited as vouchers in the U.S. National Parasite Collection (USNPC No. 96483) (Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD, USA).

Table 3.1 Measurements[a] of *Uncinaria* spp. from NZSL pups, *U. hamiltoni* from *Otaria byronia* (Baylis, 1933), *U. lucasi* from *Callorhinus ursinus* (Baylis, 1947) and *U. hamiltoni* from *Otaria flavescens* (Berón-Vera *et al.*, 2004)

	Uncinaria spp. from *Phocarctos hookeri* (present study)		*Uncinaria hamiltoni* from *Otaria byronia* (Baylis, 1933)		*Uncinaria lucasi* from *Callorhinus ursinus* (Baylis, 1947)		*Uncinaria hamiltoni* from *Otaria flavescens* (Berón-Vera *et al.*, 2004)	
	Females	Males	Females	Males	Females	Males	Females	Males
Sample size	11	8	3	?	?	?	30	29
Body length	10.35± .56 7.20-12.30	6.82± .49 5.00-9.20	12.5-17.5	8.5-12.0	12.4-16.0	7.4-8.7	11.37± 2.94 5.36-17.2	7.85± 1.52 4.88-10.64
Buccal capsule length	0.23±.004 0.22-0.25	0.20±.004 0.19-0.21	0.32-0.38	0.28-0.30	0.24-0.28	0.21-0.24	0.28±.03 0.21-0.34	0.24±.02 0.21-0.28
Buccal capsule width	0.19±.007 0.16-0.23	0.17±.005 0.15-0.19	-	-	-	-	0.24±.02 0.20-0.31	0.19±.06 0.14-0.24
Teeth height	0.044±.007 0.037-0.050	0.030±.002 0.025-0.035	-	-	-	-	-	-
Oesophagus length	1.07±.021 0.99-1.16	0.91±.021 0.86-0.99	1.60-1.90	1.50-1.55	1.25-1.40	1.10-1.20	1.15±.11 0.82-1.34	1.09±.19 0.79-1.83
Diameter of oesophageal bulb	0.18±.003 0.17-0.20	0.16±.004 0.14-0.18	-	-	-	-	-	-
Nerve ring from anterior end of body	0.69±.020 0.56-0.78	0.52±.002 0.37-0.74	0.60-1.10		0.57-0.62		-	-
Spicule length	-	0.69±.016 0.62-0.74	-	1.000	-	500-560	-	0.89±.09 0.57-1.05
Accessory piece length	-	0.060-0.080	-	0.12-0.14	-	80-90	-	-
Vulva to posterior end	4.01±.16 3.19-4.84	-	5.10-7.00	-	5.20-6.30	-	4.33±1.34 2.13-6.62	-
Length of tail	0.20±.007 0.16-0.23	-	0.16-0.25	-	0.21-0.25	-	-	-
Egg length	0.13-0.14	-	0.135-0.138	-	0.12-0.14	-	0.10-0.14	-
Egg width	0.072-0.081	-	0.085-0.093	-	0.080-0.088	-	0.04-0.10	-

[a] Measurements are given in millimeters; they are all ranges with additional means/standard deviations for NZSL hookworms

All hookworm specimens presented the general characteristics of *Uncinaria* spp.. (Fig. 3.1-3.9), with the typical anterior bend resulting in a dorsal buccal aperture (Fig. 3.1). The mouth opening spans about one half of the buccal capsule depth in dorso-ventral view. There are two pairs of cutting plates, one anterior and one posterior (Fig. 3.2 and 3.4); the latter almost entirely visible through the mouth opening in dorso-ventral view (Lyons and Delong, 2005). Buccal capsule: elongate, 202.0 ± 2.0 µm long by 167.5 ± 5.3 µm wide in males and 232.5 ± 3.7 µm by 195.4 ± 6.6 µm in females. A pair of very well-developed subventral teeth (Fig. 3.3 and 3.4); length from 25 to 35 µm in males and 27 to 50 µm in females. There is an annular thickening of the wall at the base of the buccal capsule (Fig. 3.2) and the dorsal gutter of the oesophageal gland is pronounced (Fig. 3.2 and 3.4). Cephalic sensilla visible on the cuticle include two pairs of papillae and one pair of amphids surrounding the border of the mouth opening.

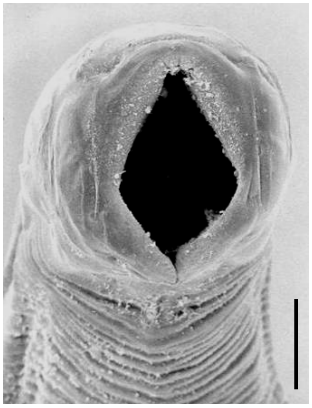


Figure 3.1 SEM of the anterior end of an adult hookworm (female) from a NZSL pup in en face view. Bar scale: 40µm

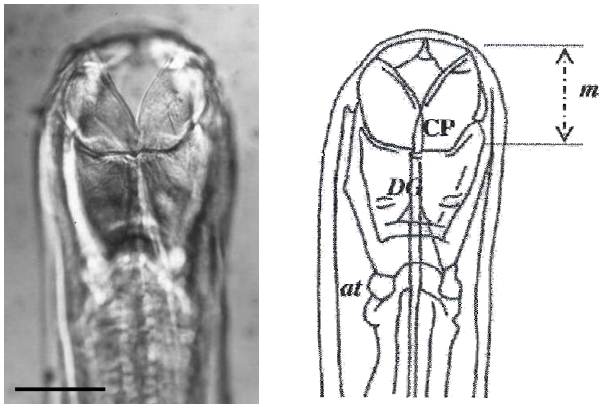


Figure 3.2 Buccal capsule of an adult hookworm (male) from a NZSL pup. Superficial dorso-ventral view showing the mouth opening (m), cutting plates (CP), dorsal gutter of oesophagus (DG) and annular thickening (at) at the base of buccal capsule. Bar scale: 80µm.

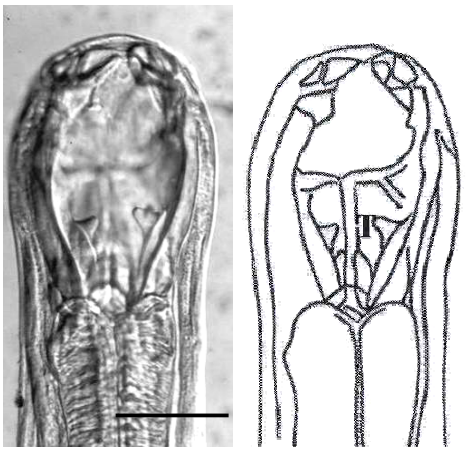


Figure 3.3 Buccal capsule of an adult hookworm (male) from a NZSL pup. Deeper dorso-ventral view underlining the pair of sub-ventral teeth (T). Bar scale: 80µm

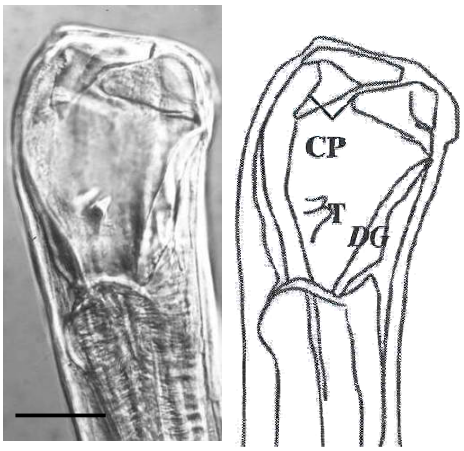


Figure 3.4 Buccal capsule of an adult hookworm (male) from a NZSL pup. Lateral view with the pair of teeth (T), cutting plates (CP) and dorsal gutter (DG). Bar scale: 80µm

Oesophagus: flask shaped, common to the other species of *Uncinaria*. Length (measured from anterior to posterior end): 912.2 ± 21.1 µm in males and 1,074.7 ± 20.7 µm in females; width at base 157.5 ± 4.1 µm in males and 183.8 ± 2.9 µm in females. Nerve ring surrounding the oesophagus above the bulb region, 526.2 ± 70.4 µm from the anterior end of the body in males and at 685.5 ± 22.5 µm in females.

Females: 10.35 ± 0.56 mm long. Vulva prominent, 4.01 ± 0.16 mm from posterior end. Vagina short and wide. Ovejectors longitudinal, symmetrical, with a combined length of 450 µm. Tail (anal opening to tail tip): 196.2 ± 6.8 µm long (Fig. 3.5). Tail mucro (Fig. 3.6): usually bent, between 10 and 30 µm long. A pair of caudal papillae at 38-40 µm from the terminal tip. Eggs: 136.6± .7 µm long by 77.1±1.4 µm wide.

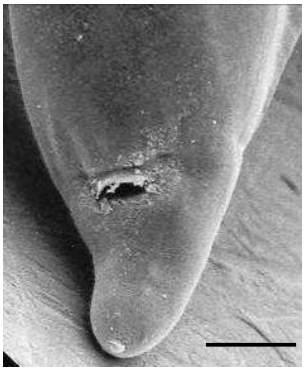


Figure 3.5 SEM of ventral view of the tail of a female adult hookworm showing the anus. Bar scale: 50µm

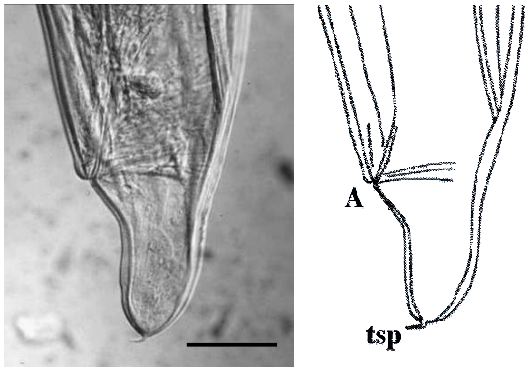


Figure 3.6 Terminal end of a female adult hookworm from a NZSL pup. Lateral view of the tail showing the anus (A) and the mucro or terminal spike (tsp). Bar scale: 80µm

Males: 6.82 ± 0.49 mm long. The two copulatory spicules are equal in length (688.5 ± 16.3 µm). Gubernaculum 60 to 80 µm long, oblong, widens posteriorly. Bursa (Fig. 3.7) with features of Order Strongylida (Olsen, 1974; Schmidt and Roberts, 1989). Ventral rays fused measuring from one half to two thirds of the medio-lateral ray (Fig. 3.8). Ventral rays diverge perpendicularly from antero-lateral ray axis (Fig. 3.8). Tips of postero- and antero-lateral rays directed away from the medio-lateral ray. Antero-lateral ray not touching edge of lateral lobe. Dorsal ray bifurcates distally with three small branches at tips, outer ray diverging. Dorsal lobe very small and semicircular. Externo-dorsal ray long, reaching margin of lateral lobe; diverges proximally from dorsal ray. Paired caudal papillae on lateral lobe.

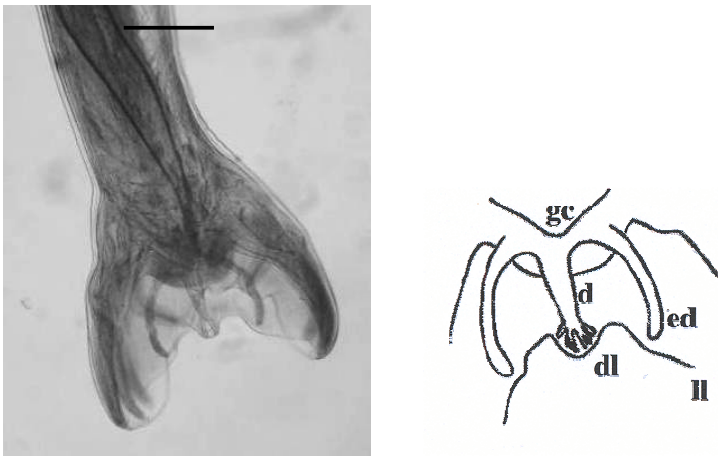


Figure 3.7 Terminal end of a male hookworm from a NZSL pup. Dorso-ventral view of bursa showing paired spicules (sp), genital cone (gc), dorsal (dl) and lateral (ll) lobes, externo-dorsal (ed) and dorsal (d) rays. Bar scale: 150µm

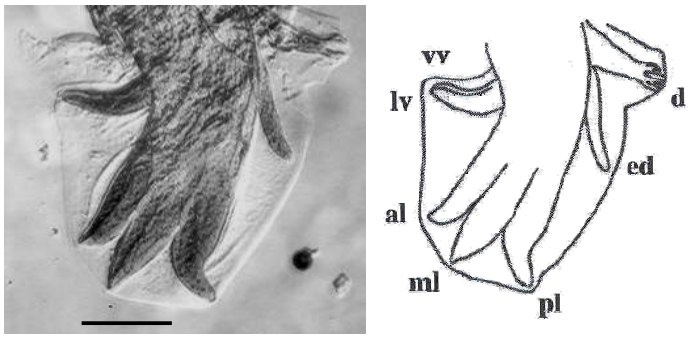


Figure 3.8 Terminal end of a male hookworm from a NZSL pup. Lateral view of copulatory bursa presenting the arrangement of ventro-ventral (vv), latero-ventral (lv) antero-lateral (al), medio-lateral (ml), postero-lateral (pl), externo-dorsal (ed) and dorsal (d) rays. Bar scale: 100µm

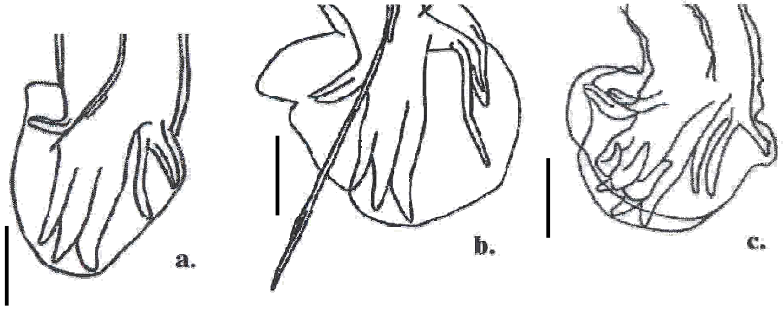


Figure 3.9 Lateral views of male copulatory bursae from *Uncinaria* spp (redrawn): (**a**) *U. hamiltoni* from Baylis (1933); (**b**) *U. lucasi* from Baylis (1947); **(c**) *U. hamiltoni platensis* from Botto and Mañe-Garzon(1975). Bar scale: 200µm

Results of statistical analyses are presented in Table 3.2 for both crude variables and ratios of the same parameters over the body length. There was a significant difference between males and females in body length ($p<0.001$), buccal capsule length ($p<0.001$) and width ($p<0.001$), oesophageal length ($p<0.001$), diameter of oesophageal bulb ($p<0.001$) and distance of nerve ring to anterior end ($p<0.001$). Tests comparing ratios of these variables to the total body length showed that gender had no effect on the position of the nerve ring ($p= 0.63$); although increasing towards the level of statistical significance, all other p-values for these size characters were still supporting differences between males and females. This suggests that ratios related to the body length could correct the bias caused by the variations of size in the specimens examined.

Variables	Difference between sexes (p-value) for crude variables	Ratio to body length		Difference between sexes (p-value) for ratio to body length
		Male	Female	
Body length	.00015	-	-	-
Buccal capsule Length	.0000013	.020-.033	.019-.033	.0212
Buccal capsule width	.00657	.017-.032	.017-.022	.000576
Oesophagus Length	.000361	.097-.173	.084-.151	.00417
Diameter of oesophageal bulb	.0000673	.027-.030	.014-.023	.00920
Nerve ring to anterior end of body	.015	.060-.088	.053-.096	.6259
Spicule length	-	.077-.113	-	-
Vulva to posterior end of body	-	-	.346-.442	-
Tail	-	-	.014-.026	-

Table 3.2 Effects of gender on measurements of *Uncinaria* spp. from NZSL pups

DISCUSSION – *Chapter 3*

Measurements of *Uncinaria* spp. from NZSL pups showed a strong sexual dimorphism (body length, buccal capsule length and oesophageal length) but there were some morphological features of the buccal capsule and the oesophagus that did not differ between sexes. Likewise, gender had no effect on the position of the nerve ring when statistical comparison included the ratio of the distance as based on total body length. This indicates that ratios of certain measured variables may provide more accurate conclusions, than from those based on specific measurements, especially when comparing *Uncinaria* spp. in pinniped hosts (George-Nascimento et al., 1992) where sexually mature hookworms can vary substantially in total body length. Ranges of body length in *Uncinaria* spp. from NZSL were similar to those from Berón-Vera et al. (2004) but were reduced relative to *U. lucasi* and *U. hamiltoni* as described by Baylis (1933, 1947). Almost all variables measured in hookworms from the southern hemisphere (Botto and Mañé-Garzón, 1975; Berón-Vera et al., 2004) present a broad range with regard to measurements important for comparing species: buccal capsule length, oesophagus length, distance of vulva to posterior end, spicule length and egg dimensions (Table 3.1). Disparity in the size of mature hookworms within the same host was observed during the present study. The measurements from *Uncinaria* spp. found in the northern hemisphere show less variation (Nadler et al., 2000). Lyons (2005) quotes Olsen (1952) "While the body size of the hookworms from the sea lions tends to be larger than those from the fur seals, the relative proportions on a percentage basis remain almost the same". This pertains to *U. lucasi*, from northern fur seal (*Callorhinus ursinus*) and Steller sea lion (*Eumetopias jubatus*) pups from St. Paul Island, Pribilof Islands, Alaska (Olsen, 1952). Using ratios may permit compensation for this type of bias. It seems inappropriate to include body width in comparisons for *Uncinaria* spp. in NZSL, not only because mounting specimens between a slide and a cover slip is highly likely to deform the diameter of a nematode, but because previous authors do not identify the precise site of body width measurement.

CONCLUSION – *Chapter 3*

The arrangement of the bursal rays of *Uncinaria* spp. in the New Zealand sea lions is characterized by the configuration of lateral rays resembling *U. lucasi* and the externo-dorsal ray being much longer than the dorsal one is a feature more similar to *U. hamiltoni.* This reinforces the need for an extensive morphometric examination of *Uncinaria* species in pinnipeds.

Chapter 4

Influence of climatic conditions on the epidemiology of hookworm infection in New Zealand sea lions (*Phocarctos hookeri*) at Sandy Bay Beach, Enderby Island (Auckland Islands).

INTRODUCTION – *Chapter 4*

The New Zealand sea lion (NZSL), *Phocarctos hookeri* is an endemic and endangered species that almost exclusively breeds on the Auckland Islands (between latitudes 48ºS and 53ºS) every austral summer, from December to February. The climatic conditions in this Sub-Antarctic region are characterized by persistent westerly winds and abundant precipitations occurring as showers. The maximum air temperature ranges between 10 and 16 ºC in summer and between 4 and 10 ºC in winter. Enderby Island usually has the finest and warmest weather of all the Auckland Islands (Peat, 2003).

During the 1998/1999 to 2004/2005 breeding seasons, the annual mortality at Sandy Bay rookery from birth to two months of age was about 10%, which is similar to what was reported in other pinniped species for the same age group (Baker and Baker, 1988; Aurioles and Sinsel, 1988). The main causes of pup mortality were identified and described in Chapter II. These were trauma, bacterial infection, starvation, hookworm infection and stillbirth. Some of these causes are likely to be under the influence of climatic fluctuation. For instance, severe weather conditions (storms, heavy rain and strong winds) were often associated with pups' dying from hypothermia and drowning over the period of study. Climatic fluctuations can also influence pup health and survival by modifying the food stock available to lactating females around the Auckland Islands for lactating females (Chilvers et al., 2006b) or by killing hookworm free-living stages (Castinel et al., submitted *a*; *see* Chapter 5). For example, it was assumed that a decline in prey available for northern elephant seal females, prior to parturition, had negatively impacted on the mean weaning mass of their pups (LeBoeuf and Crocker, 2005).

The effect of temperature on the free-living stages of hookworms can be estimated directly using temperature recorders buried beneath the surface of the beach, where the free-living phase of the life cycle takes place (Lyons, 1963). Pups shed faeces contaminated with *Uncinaria* spp. eggs on the sand surface and these will hatch into ensheathed and mobile larvae (Castinel et al., submitted *a*; *see* Chapter 5).

The aim of this chapter is to report on some of the climatic conditions occurring at Sandy Bay Beach rookery during the breeding seasons from 2000/2001 to 2003/2004 and to discuss the possible effect of temperature variations on hookworm free-living larvae on the rookery.

Air temperature monitoring

Air temperatures and other atmospheric parameters are accurately recorded on Enderby Island all year round by a climate station established by the National Institute of Water and Atmospheric Research (NIWA Ltd, Wellington, New Zealand). This is slightly inland and on an elevated site (Fig. 4.1). NIWA provided hourly temperature records of December, January and February for the years 2000/2001 to 2003/2004.

Figure 4.1 NIWA climate station on Enderby Island.

Soil temperature monitoring

Soil temperature was recorded hourly with two Hobo data loggers (Hobo H8, Scott Technical Instruments, Hamilton, New Zealand) buried 20 cm deep on Sandy Bay beach during January and February 2003/2004, at two different sites on Sandy Bay beach. They were located 150 metres apart, at the periphery of the breeding colony and only a few metres away from the sea lions. Temperature records were analysed with the BoxCar software (Version 3.7.3, Onset computer Corp. 1995-2002).

Statistical analyses

Statistical analyses were performed on pairs of minimum and maximum temperatures to compare records between the two sites for the 2003/2004 season only. Datasets were compared using the ANOVA procedure in SAS (Statistical Analyses System® 9.1.3,

SAS Institute Inc., Cary, NC, USA). The daily amplitude (difference between maximum and minimum temperatures) was compared between the beach records and those from the NIWA station, using the ANOVA procedure as described above. A statistical difference was accepted when $P<0.05$.

Linear regression equations, for both minimum and maximum temperatures, were obtained after plotting the daily temperatures recorded on the beach in 2003/2004 (pooled sites) against their paired NIWA records for the same period. This will allow extrapolating temperatures for the previous seasons using the NIWA archives.

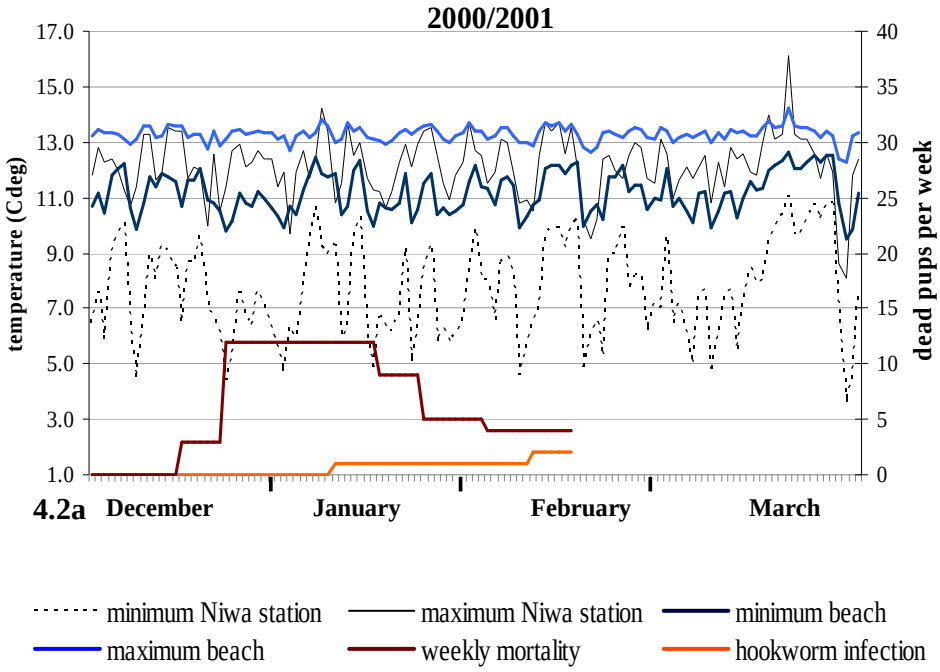
The daily temperatures monitored at the two different sites on Sandy Bay beach did not significantly vary in 2003/2004, for both the minimum and the maximum records. As a consequence, data were pooled for both sites for minima and maxima to facilitate comparisons between years. NIWA records for 2003/2004 were plotted against these pooled data. The linear regression equations for minimum (*Y=0.4234*X+7.9502*) and maximum temperatures (*Y=0.2445*X+10.318*) were used to calculate the beach temperatures from NIWA archived data for the breeding seasons prior to 2003/2004. "*Y*" was the beach daily minimum or maximum, and "*X*" the NIWA record. The summary of extrapolated temperatures is presented by months and by years in Table 4.1. Figures 4a to 4d show the daily estimated records of temperatures on the beach and from NIWA climate station for 2000/2001, 2001/2002, 2002/2003 and 2003/2004 respectively. The margin between daily minimum and maximum temperatures ranged between 0.6 and 4.2 ºC and between 0.5 and 5.4 ºC in 2003/2004. This contrasted with the NIWA daily records which amplitude was significantly greater than the two other sites ($P<0.0001$).

breeding season	**D**ecember	**J**anuary	**F**ebruary	**M**arch
2000/2001	11.0 - 13.3*	11.1 - 13.3	11.3 - 13.3	11.3 - 13.3
2001/2002	11.4 - 13.4	11.8 - 13.5	11.6 - 13.6	10.9 - 13.1
2002/2003	11.0 - 13.3	11.4 - 13.4	11.1 - 13.3	11.4 - 13.2
2003/2004	11.1 - 12.5	11.8 - 13.9	10.6 - 12.0	N/A

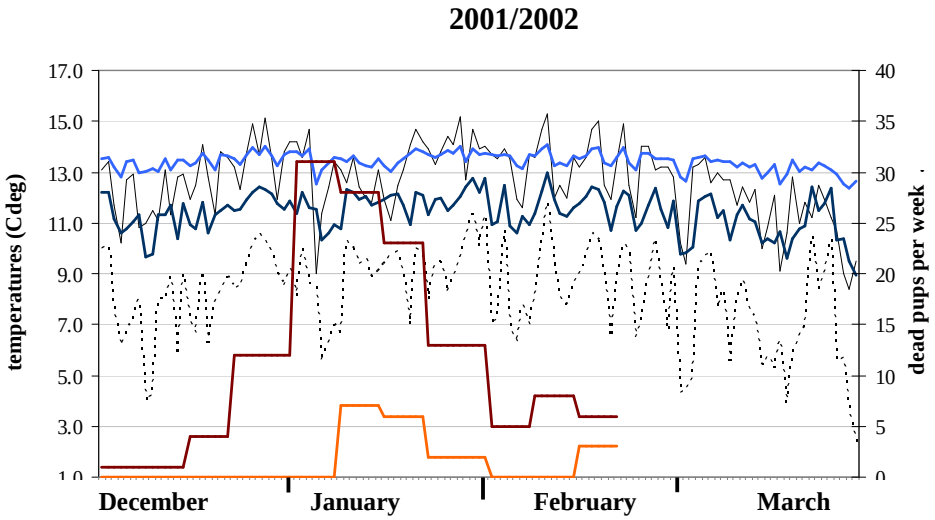
*minimum temperature-maximum temperature

Table 4.1 Minimum and maximum temperatures (ºC) by months for the 2000/2001 to 2003/2004 breeding seasons.

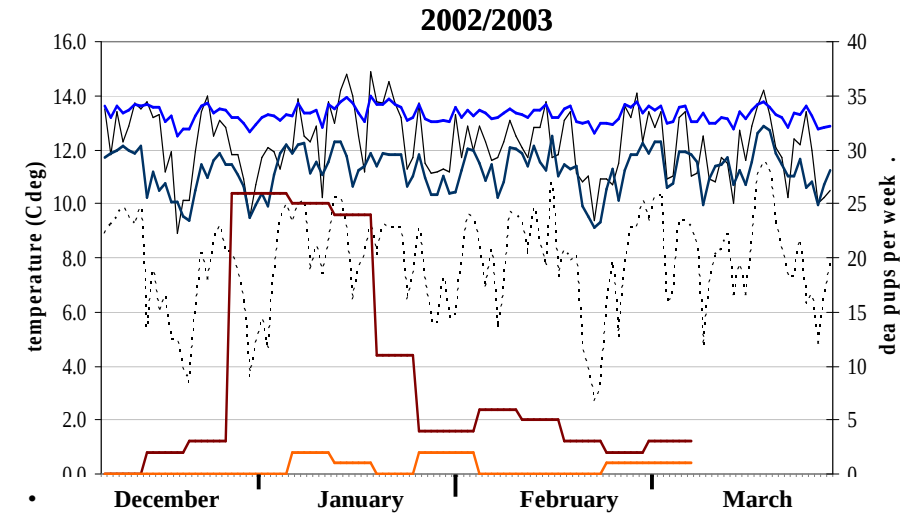
Figure 4. 2 (a, b, c, d) Temperatures recorded from December to March, at two different sites on Enderby Island in 2000/2001 (a), 2001/2002 (b), 2002/2003 (c) and 2003/2004 (d). The graphs also show the number of dead pups per week (weekly mortality) and the number of pups diagnosed with hookworm infection as a primary cause of death per week (hookworm infection). The bold arrow on the 2003/2004 graph indicates the time of pup faeces first found positive for *Uncinaria* spp. eggs.



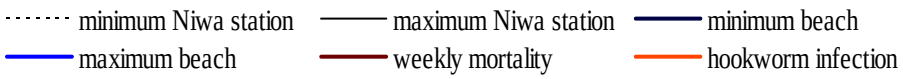
4.2a

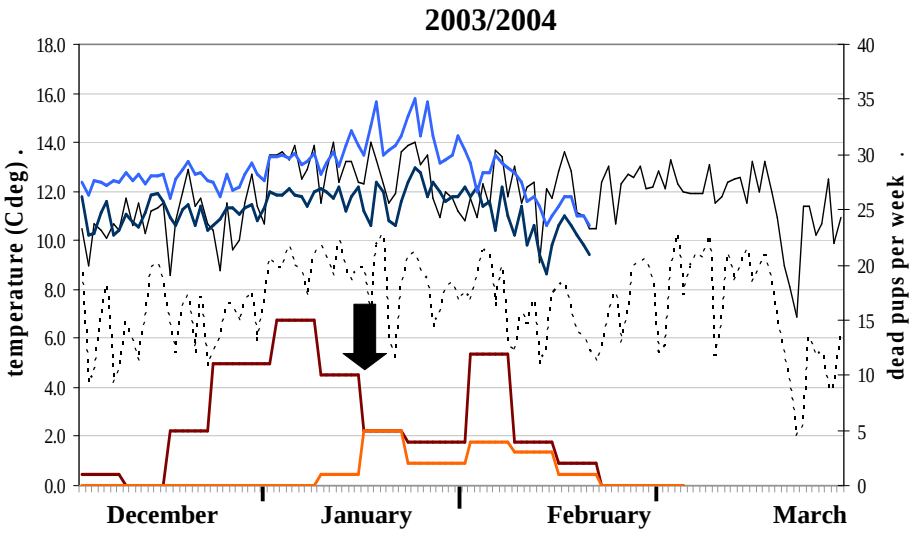


4.2b


2002/2003
temperature (Cdeg)
dea pups per week .
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
40
35
30
25
20
15
10
5
0
December
January
February
March

4.2c


minimum Niwa station
maximum Niwa station
minimum beach
maximum beach
weekly mortality
hookworm infection


2003/2004
temperature (Cdeg) .
dead pups per week .
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
40
35
30
25
20
15
10
5
0
December
January
February
March

4.2d

DISCUSSION – *Chapter 4*

There was a marked difference in the daily variations between the NIWA climate station and the beach. This may be linked to the experimental conditions of recording. Firstly, the station is located at a higher altitude and on a flat area, directly exposed to the strong winds. In contrast, Sandy Bay beach, where the Hobo sensors were buried, was relatively sheltered. Secondly, the NIWA station measures air temperatures whereas the data loggers recorded soil temperatures at 20 cm under the surface. The reason why these were not set up on the surface of the beach was because of the intense activity taking place on the rookery during the breeding season. This comprised bull fights and pups' extreme curiosity when they wander on the beach while waiting ashore for their mother. Also, to attenuate heat intake due to sun exposure, sea lions very often throw large amounts of sand with their flippers onto themselves. Consequently the surface of the beach around the harem is constantly being turned over. In addition, occasional storms with strong winds and rain could have easily swept away the recording instruments if located on the surface. Given that development and hatching of hookworm eggs take place in the faecal matter deposited on the sand by infected pups, the temperatures recorded in this experiment may not strictly reflect the conditions in which hookworm free-living stages develop. However, once deposited on the soil surface, faeces were soon covered with sand as a result of sea lion activity on the rookery ground.

According to Beaver (1953) and Vinayak et al. (1979), optimal conditions for the development of hookworm larvae are sandy soil with sufficient moisture to protect developmental stages from fatal desiccation (Cort, 1925; Beaver, 1953). A notable feature of the temperatures recorded and estimated over the seasons in the current study is how stable the summer temperatures were from year to year. Although relatively cool, the daily maxima only varied between 12.0-13.9ºC and the daily minima were only two to three degrees below. Such a stable temperature is likely to be beneficial to hookworm larval development and survival. These conditions are more likely to be met at 20 cm-deep than on the surface. The capability of free-living larvae to survive severe climatic conditions is intrinsic to a nematode species (Boag and Thomas, 1985) and

Uncinaria species showed that they were capable of developing at lower temperatures than *Ancylostoma* species (Hill and Roberson, 1985). The reasons for investigating temperatures on Sandy Bay beach were to establish the conditions in which hookworm larvae were likely to hatch, and to some extent, hypothesize on the likelihood for these larvae to survive over the winter in the soil at the rookery and infect the next season pups. Olsen (1958) studied the survival of *Uncinaria lucasi* eggs and larvae at different temperatures on the Pribilof Islands in Alaska. From these experiments, it appeared that free-living larvae could survive for a few weeks at 44-46ºF (approximately 7-8ºC) but would die after 24 hours at 24-26 ºF (approximately minus 4ºC) and at minus 4ºF (minus 20ºC). Summer temperatures at Sandy Bay Beach were not as low as those recorded in Alaska but winter data for Enderby Island were not available. Therefore it was not possible to determine whether the absence of hookworm larvae in the soil at Sandy Bay Beach rookery early in the summer season was subsequent to negative temperatures occurring during the previous winter. Also, larvae were recovered in great numbers in the sand on the Pribilof Islands in spring, before northern fur seal pups were present on the rookery, which suggested that larvae had survived through the winter for that year (Olsen, 1958). In contrast, hookworm larvae were not recovered from soil samples before the 16th of January 2004 and the 17th of February 2005 at Sandy Bay Beach rookery (*see* Chapter 5), indicating that free-living larvae were not present in the sand before NZSL pups started shedding eggs in their faeces. Whilst Olsen (1958) deducted that each generation of northern sea lion pups was infected by larvae hatching from eggs deposited by pups during the preceding season, this does not seem to apply to the situation at Sandy Bay Beach. Besides the one-year study on northern fur seals in Alaska (Olsen, 1958), further observations were later conducted over several seasons on California rookeries shared by both California sea lions and northern fur seals (Lyons et al., 2000a). This study stressed that seasonality occurred in larvae hatching but it also diverged from Olsen's hypothesis that larvae overwintered to infect pups of the following generation. Lyons et al. (2000a) found larvae in the sand in both autumn and winter months but not in the following summer, when pups were born. Climatic data were not reported for these particular studies (Olsen, 1958; Lyons et al., 2000b) but it is likely to be colder in Alaska (Olsen and Lyons, 1965) and on the Auckland Islands (present study) than in California. It is clearly very difficult to compare the influence of temperature alone on overwinter survival of *Uncinaria* spp. larvae. However,

determining if it is possible is important for understanding the hookworm life cycle (*see* Chapter 5).

Another factor which may play a role in larval survival is the substrate in the environment. The configuration of Sandy Bay beach changes radically from summer to winter (Fig. 4.3 and 4.4). Over winter, the sand is almost entirely washed away and underlying rocks become exposed to the waves. Hookworm free-living stages are likely to be physically washed away with the sand when it disappears in the winter. As a consequence, it is very unlikely that any hookworm larvae could survive both low winter temperatures and the removal of the sandy soil on Sandy Bay Beach. Over-wintering and the hookworm life cycle in NZSLs is studied in more detail in Chapter 5.

Figure 4.3 Sandy Bay Beach rookery (Enderby Island) in summer months.

Figure 4.4 Sandy Bay Beach rookery (Enderby Island) in winter months.

CONCLUSION – *Chapter 4*

In summer months, daily temperatures on Sandy Bay beach seemed particularly stable beneath the sand surface. Therefore, some factors, more likely to be intrinsic to the hookworm species rather than related to environmental temperatures, may explain the incapacity of free-living larvae to survive on the rookery over the winter. This represents an important feature to understand how NZSL pups become infected with *Uncinaria* spp. The mode of infection and the entire hookworm life cycle will be further discussed in Chapter 5.

Chapter 5

Epidemiology of hookworm (*Uncinaria* spp.) infection in New Zealand (Hooker's) Sea Lion (*Phocarctos hookeri*) pups on Enderby Island (Auckland Islands) during the breeding seasons from 1999/2000 to 2004/2005.

Submitted for publication
to *Parasitology Research*

ABSTRACT – *Chapter 5*

This is the first investigation of the epidemiology of hookworm (*Uncinaria* spp.) infection in New Zealand sea lions (NZSLs) (*Phocarctos hookeri*) on Enderby Island, Auckland Islands. Examination of faeces for hookworm eggs in various age categories of sea lions revealed that only pups up to at least three months of age harboured adult hookworms in their intestines. Gross necropsy of more than 400 pups from 1999/2000 to 2004/2005 showed that the prevalence of hookworm infection varied significantly between years and was higher from mid-January to the end of February, when the majority of pups were between three and nine weeks old. The average burden of adult parasites per pup was not influenced by the host's sex and body condition or by year. This study also provided evidence for transmission occurring by the transmammary route in NZSLs.

Adult hookworms (*Uncinaria* spp.) have been reported frequently in otariids from a wide geographical area. So far, the taxonomy refers to two species formally described for this host family: *Uncinaria lucasi* and *Uncinaria hamiltoni* (Stiles, 1901; Baylis, 1933, 1947). However, specimens presenting morphologic differences and intermediate measurements have also been reported (Dailey and Hill, 1970; Nadler et al., 2000; Castinel et al., 2006) suggesting the existence of more than two species of hookworms infecting otariids world-wide. Despite a single report of adult hookworms in the intestines of one sub-adult male Steller's sea lion (*Eumetopias jubatus*) (Olsen, 1958), all other reports have described the parasitic infection almost exclusively in otariid pups (Lucas, 1899; Lyons, 1963; Dailey, 1975).

Lucas (1899) collected the very first data on the prevalence of hookworms in the northern fur seal (*Callorhinus ursinus*). He described *Uncinaria* spp. infection as the most important factor in pup mortality on Saint Paul Island (SPI) (Pribilof Islands, Alaska) for the summer 1898/1899, when 61% of the pups were diagnosed with hookworm infection as the primary cause of death. Lyons (1963) likewise reported a high prevalence of hookworms (between 56 and 85%) in *C. ursinus* on SPI for the years 1960 to 1962. The burden of adult parasites recovered from otariid pups has been reported to be highly variable and influenced by both the host species and the rookery (Lyons et al., 2001, 2005). On SPI, neonatal mortality associated with hookworms in northern fur seals was reported to be higher on sandy rookeries than on non-sandy ones (Lucas, 1899; Olsen, 1958) indicating that the type of substrate could play a role in the epidemiology of these parasites. Supporting the hypothesis of spatial variability of infection intensity, Hughes et al. (2004) reported a difference of parasitic burden in Steller's sea lion pups between two different sites, with about 8 times more hookworms per individual on the Southeast Alaskan Hazy Island than on Forrester Island. In addition, Lyons et al. (2005) showed that infection intensity in California sea lion (*Zalophus californianus*) pups on San Miguel Island (California) was generally higher in pups with good body condition and in males. This underlined the existence of individual variations within the same population and the possibility of some categories of pups being more susceptible to hookworm infection than others.

The life cycle of *Uncinaria lucasi* was described in northern fur seals by Lyons (1963) and Olsen and Lyons (1962, 1965) on the Pribilof Islands. The route of infection of fur seal pups was found to be passage of the larvae through the mammary system to nursing offspring with resultant maturation to an adult parasite in the small intestine of the pup as it occurs in domestic dogs infected by *Ancylostoma caninum* (Burke and Roberson, 1985). The cycle was divided into three parts: (1) the free-living phase, with eggs developing into third-stage larvae (L_3) in the soil; (2) the tissue phase, with parasitic L_3 found in the hosts' tissue; and (3) the intestinal phase, with the ingestion of parasitic L_3 in the first milk and development into adult hookworms in the intestines of pups. The characteristic transmammary transmission described by the authors on this occasion in northern fur seals was also reported for *Uncinaria* spp. in Juan Fernandez fur seals (Sepúlveda and Alcaíno, 1993) and in California sea lions (Lyons et al., 2000a). Several additional studies have compared measurements of larvae found in different tissues from animals of various ages (Olsen and Lyons, 1965; Lyons and Keyes, 1978; Sepúlveda and Alcaíno, 1993; Lyons et al., 2003). Larval sizes varied considerably depending on both the host's age and the tissue sampled, with larvae from the mammary glands of lactating females being longer than those found in blubber of other adult fur seals. Recovery of *Uncinaria* spp. larval stages from the soil revealed that free-living hookworm L_3 were ensheathed, were shorter than those found in the host's tissue (Olsen and Lyons, 1965; Lyons et al., 2000b), and sometimes could survive the winter temperature on the rookery (Olsen, 1958).

The New Zealand sea lion (NZSL) is one of the world's rarest and most highly localized pinnipeds. NZSLs breed on New Zealand's subantarctic Auckland Islands between latitudes 48°S and 53°S (Gales and Mattlin, 1997). Following a large number of deaths due to an epidemic of unknown aetiology in 1997/1998, a surveillance program was established to monitor in detail all mortality events in the population at Sandy Bay. Since the 1998/1999 breeding season, all pups that died during that time at this location have been necropsied in the field to determine cause of death. During the survey, two successive seasons (2001/2002 and 2002/2003) were marked by an unusually high mortality of pups, which was attributed to the pathogen *Klebsiella pneumoniae* (Wilkinson et al., 2006). *Uncinaria* spp. (Castinel et al., 2006) was

diagnosed as the primary cause of death for 13% of pups and was a consistent finding between years (Castinel et al., submitted *b; see* Chapter 2).

This study is the first investigation of the epidemiology of hookworm infection in NZSL with emphasis on the prevalence and life cycle of *Uncinaria* spp. in pups born on Sandy Bay Beach (Enderby Island) during the breeding seasons from 1999/2000 to 2004/2005.

Faecal examination for hookworm eggs

Faecal samples (~ 1 gram each) were randomly collected from live animals (both adults and pups) on Sandy Bay Beach (Enderby Island, Auckland Islands) from mid-January to mid-February 2004 and from early December 2004 to mid-February 2005. NZSL females give birth over a period of less than a week with the annual peak of birth occurring on about the 26th of December (Chilvers et al., 2006a). Therefore the age of the pups during the seasonal studies ranged from birth to two to three months of age. Various age classes were represented. In total 200 pups plus 30 adults and sub-adults were sampled for faeces over the two seasons. In 2004/2005, as part of a growth study, some pups born to known females were identified at birth and were paired by sex and age. In order to investigate the mode of infection, one pup from each pair was given a subcutaneous injection of ivermectin (200 µg/kg; Ivomec® injection for cattle, sheep and pig, 10g/L ivermectin, Merial New Zealand Ltd) at two days, one week, and one month of age (n=24) or just at two days old (n=5). All marked pups were sampled for faeces with a rectal loop either weekly or fortnightly. Examination of faecal samples consisted of a direct smear if only a small amount of material could be recovered, otherwise of flotation with saturated NaCl solution followed by microscopic examination.

General necropsy procedures

As part of a monitoring programme from 1998/1999 to 2004/2005, all sea lions found dead on Sandy Bay Beach each year from early December through the end of February were necropsied following a standard protocol (Castinel et al., submitted *b*; *see* Chapter 2). This included 455 pups, six yearlings, two sub-adults, and 21 adults. Samples were collected for histopathology, serology, parasitology, bacteriology, virology, genetics, and life history studies; but only data on hookworms are presented here. Morphological characteristics were systematically measured as follows: body length and girth (in meters), abdominal blubber depth immediately posterior to the umbilicus (in millimeters), and body weight (in kilograms). The body condition was rated in accordance with the abdominal blubber depth (poor: less than 5 mm, moderate: 5 to 10 mm, fair to good: 10 to 15 mm, or excellent: more than 15 mm).

Specific necropsy examination of intestinal tracts for hookworms

From 1999/2000 to 2002/2003, 80 intestinal tracts were collected from pups at necropsy and fixed in 10% buffered formalin, to be later examined at Massey University (Palmerston North, New Zealand). In order to assess the hookworm burden, the intestines were first opened over their whole length and flushed with running water. When hookworms were too deeply anchored, they were removed with forceps. The contents were then filtered successively through a 250-µm mesh and a 50-µm mesh sieves and both retentates were examined. In 2003/2004 and 2004/2005, fresh intestines of 28 pups, three yearlings, and six adults were processed on site using the same technique. Among the 108 pup intestines examined from 1999/2000 to 2004/2005, 17 were divided into three equal segments for the small intestine (proximal, medial and distal) and one segment for the large intestine starting at the caecum. For these pups, the worm burden was counted separately for each portion. In the present study, the prevalence is reported for each breeding season as follows: the overall prevalence at post mortem (P), the proportion of infected pups at necropsy from the start of the pupping season in December to the 14th of January (P1), and the proportion from the 15th of January to the end of February (P2). Assuming infection occurred immediately after birth, P1 was expected to cover the prepatent period whereas P2 would correspond to the clinical phase of hookworm infection.

Specific necropsy examination of fresh tissues for parasitic hookworm larvae

In 2003/2004 and 2004/2005, various fresh tissues were collected to search for parasitic larval stages from the placenta, ventral abdominal blubber and the mammary gland. Ventral abdominal blubber was sampled at necropsy only in freshly dead animals (15 pups, one adult male, one female yearling, and three adult females). A sample of approximately 10 cm^2 area and full blubber depth was taken 2 cm posterior to the umbilicus in each animal. The entire mammary glands were collected from the three adult females. In the yearling female, the mammary gland was not developed enough to be separated from the blubber and the sample from this animal included a mix of both tissues. Seven fresh placentas were collected immediately after expulsion. Each tissue sample was immediately processed for larval recovery using a Baermann apparatus (Baermann, 1917). This was modified as follows: tissues were cut into pieces no larger

than 1 cm^3, a 10 cm diameter plastic funnel was connected by rubber tubing to a sterile 15-ml tube, the material to be analysed was suspended inside two gauze swabs on top of the cone, and both tube and funnel were filled with warm water (20-25ºC) until tissues were submerged. The installation was left at ambient temperature (approximately 14 to 17ºC) for six hours and the sediment from the bottom content of the tube was examined under a stereo-microscope at magnification ranging from 10 to 35 times.

Examination of stomach contents of pups for infective hookworm larvae

Small volumes of milk (2 - 5 ml) were drawn from the stomach of three live pups by means of a 50-ml disposable syringe connected to a flexible plastic tube (1 cm diameter, 0.5 to 1 meter long, depending on the age of the pup). Each pup was sampled three times: on its day of birth plus three and seven days later. The stomach contents were processed with the modified Baermann method as previously described. The milky material mixed with the water in the funnel, but any solid particles were retained by the gauze swabs. Faeces were also collected with a rectal loop on the same days and occasionally until these pups were three weeks of age.

Egg culture and in vitro study of larval development

Three faecal samples, heavily contaminated with hookworm eggs, were placed in a Petri dish and mixed with water. The milieu was then oxygenated with air bubbles and left at ambient temperature that was recorded using a data logger (Hobo H8, Scott Technical Instruments, Hamilton, New Zealand), also used to monitor the soil temperature on the beach.

Soil sampling and temperature recording to investigate the free-living phase

Soil cores were removed with a steel T-shaped corer at different sites on the beach in 2003/2004 and 2004/2005. Each sample consisted of approximately 140 cm^3 of sand. In parallel, soil temperature was recorded hourly in January 2003/2004 and from mid-December to the first week of February in 2004/2005 with a data logger buried 20 cm deep on the beach, only a few meters from the harems.

Statistical analyses

The general prevalence of hookworms at necropsy and hookworm infection as a primary cause of mortality were analysed using the GENMOD procedure in the statistical computer package SAS (Statistical Analyses System® 9.1.3, SAS Institute Inc., Cary, NC, USA) with a logit model that considered the fixed effects of year, sex, body condition, time in the season (P1 and P2) and their interactions. For the variable hookworm infection as a primary cause of mortality only, the fixed effect of the log transformed hookworm burden was also included in the model. The log transformation of hookworm burden was analysed with the MIXED procedure for which the model tested the fixed effects of year, sex, body condition and their interactions. The mean hookworm burden per individual and the mean burden by intestinal segment are presented as back-transformed least mean squares together with their back-transformed 95% confidence interval (CI). A statistical difference was accepted when $P<0.05$. Measurements of third-stage larvae and temperatures are given as means ± standard errors.

Prevalence of hookworms in pups at necropsy

Uncinaria spp. eggs were seen exclusively in preparations of pup faeces. The faecal survey showed that 75% of live pups were infected and that samples were positive for hookworm eggs from mid-January to the end of February, when the field studies ended each year. Fig. 5.1 presents the results of a faecal screening in live pups compared to necropsy findings for 2004/2005. Faeces from all examined pups were positive for *Uncinaria* spp. eggs in the last week of January and all dead pups were infected with adult hookworms from the first week of February. The first faecal sample positive for hookworm eggs was found on the 10th of January 2004 and on the 14th of January 2005, respectively, 34 and 37 days after the first birth of the season and 16 and 19 days after the seasonal peak of birth. Post treatment faecal samples from pups treated with the antiparasitic drug ivermectin were always negative for hookworm eggs, regardless of the protocol (one or three treatments).

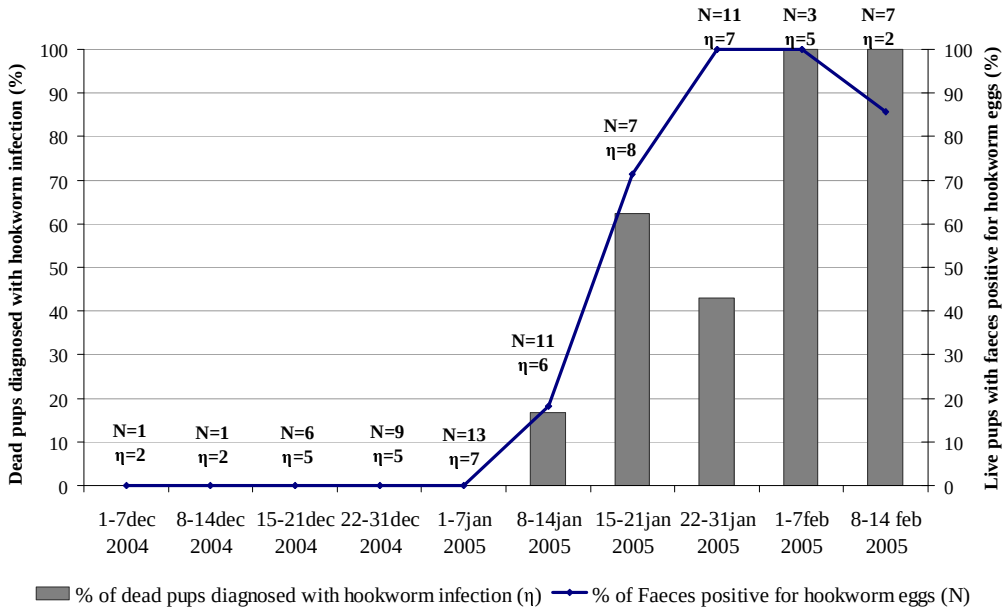


Figure 5.1 Incidence of hookworm infection in live and dead NZSL pups on Sandy Bay Beach (Enderby Island) in 2004/2005. A faecal survey was conducted in live pups and results were grouped to give weekly counts (N is the sample size). The number of pups diagnosed per week is given by ŋ. The majority of NZSL pups were born around the 26th of December.

The prevalence of hookworm infection at necropsy was based on the presence of adult specimens of *Uncinaria* spp. regardless of whether they were sexually mature (eggs in female uterus) or not. Adult hookworms were found in pups (n=455) but not in any older sea lions (n=29). Prevalence of hookworm infection varied between years with a significantly lower percentage of animals diagnosed in 2001/2002 compared to the previous season ($P<0.05$) but a higher percentage of infected pups was found at necropsy in 2002/2003 than in 2001/2002 ($P<0.05$) (Fig. 5.2). Hookworm infection as a primary cause of death was diagnosed in more pups during the second part of the study period with P2>P1 ($P<0.0001$). There were also significantly more male pups than female pups where hookworm infection was the first cause of death ($P<0.05$). As the other factors (year and body condition) and their interactions were not significant, they were removed from the analysis.

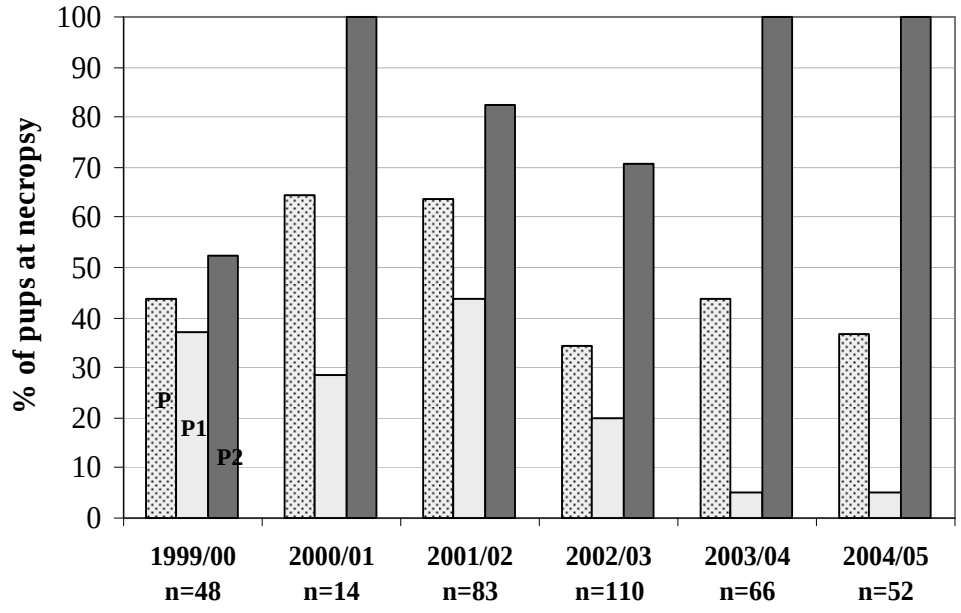


Figure 5.2 Prevalence of hookworm infection in NZSL pups at necropsy, during the breeding season, from 1999/2000 to 2004/2005**.** Post-mortem examinations were conducted from early December to the end of February each year. P is the overall prevalence for the entire period of study; P1 the prevalence to mid-January and P2 the prevalence from mid-January to the end of February. "n" is the total number of necropsy pups considered for the prevalence study.

Patterns of hookworm infection in pups at necropsy during the breeding seasons are depicted in Fig. 5.2. The seasonal prevalence of hookworm infection (P) remained stable over the years but there was a significant difference between P1 and P2 ($P<0.001$) with P2>P1. In 2003/2004 and 2004/2005, hookworms were found in less than 10% of pups at necropsy before mid-January (P1) but all were found infected afterwards (P2=100%). There was no significant effect of sex and body condition on the general prevalence of hookworm infection at necropsy.

Numbers of hookworms in different segments of small intestine of pups

The number of hookworms in intestinal segments was indicative of a preferential location of the parasite in the first two thirds of the small intestine; no specimens were recovered in the caecum or the large intestine. The back-transformed arithmetic mean and 95% CI for individual counts in the first, second and third segments of the small intestine were respectively 193 [76-489], 519 [210-1,280] and 198 [78-503] hookworms. Even though the number of parasites did not significantly differ between segments, there seemed to be more hookworms in the middle portion of the small intestine.

Hookworm burden relative to sex, year, and body condition in pups

The back-transformed mean and its 95% CI for individual burden were 824 [136-3,323] parasites. Sex had no effect on the number of parasites recovered for the period 1999/2000 to 2004/2005. However, the analysis of variance showed variations in worm burden between years, with *P* standing just within the limits of significance. Hookworm burdens for 2001/2002 (back-transformed mean of 334) were significantly lower than for 2003/2004 and for 2004/2005 with back-transformed means of 1781 ($P<0.005$) and of 1439 ($P<0.05$) respectively. None of the other factors (sex and body condition) or their interaction significantly contributed to explain the model. This strongly suggests that the variations observed for the model of hookworm burden were explained by the variability between individuals ($P<0.05$).

Recovery of hookworm larvae from sea lion tissues

Table 5.1 presents the body length of larvae recovered from the various tissue samples. In January 2003/2004, several live larvae were extracted from blubber samples

in four of five pups (no measurements were recorded but all were exsheathed); whereas, in 2004/2005, no larvae could be observed in the 10 samples collected from pups between the 6th of December 2004 and the 3rd of February 2005. No larvae were recovered from any of the seven placentas collected in 2004/2005 or from the mammary glands of two adult females (one sampled in January 2004 and the other in February 2005 when its pup was 51 days old). In contrast, some larvae were recovered in December 2004 from the abdominal blubber of an adult male, from a mixture of belly tissues of a yearling female, and from the mammary glands of a lactating adult female. The size of the larvae varied considerably depending on the source (Table 5.1). All were without sheath and showed the characteristics of parasitic third-stage larvae of hookworms (Gibbs, 1961; Lyons, 1963).

Tissue phase	**Nature of sample**	**Date (no. larvae)**	**Measurements of larvae** [range] (mean, ensheathed/no sheath)
Pups (4/15)	Ventral abdominal blubber	Jan.-Feb. 2004 (5)	Not done (no sheath)
Yearling female (1)	Ventral blubber + mammary glands	Dec. 2004 (10)	[743.6-860.2 µm] (798.2 µm, no sheath)
Adult male (1)	Ventral abdominal blubber	Dec. 2004 (2)	[701.8-717.6 µm] (709.7 µm, no sheath)
Lactating adult female (1/3)	Mammary glands only	Dec. 2004 (10)	[742.8-1046.0 µm] (894.4 µm, no sheath)
Milk from pups' stomach	**Age of pup (days)**	**no. larvae**	**Measurements of larvae**
Pup A	1	9	[840.9-1061.5 µm] (988.0 µm, no sheath)
Pup A	5	4	[807.7-1071.4 µm] (904.8 µm, no sheath)
Pup C	3	4	[1143.7-3285.7 µm] (2966.9 µm, no sheath)
Soil samples	**Sampling site**	**Date (no. larvae)**	**Measurements of larvae**
Sand	"Wendy hut"	Jan. 2004 (4)	[609.4-764.0 µm] (703.8 µm, ensheathed)
Sand	Creek	Feb.2005 (13)	[228.7-686.4 µm] (499.2 µm, ensheathed)

Table 5.1 Measurements of larvae recovered from different types of samples on Sandy Bay Beach, Enderby Island. This table shows the source and body length of larvae (minimum and maximum are given between brackets followed by the median, best representing the average length given the small sample size). For the tissue phase, the number of animals positive for larvae is compared to the number of individuals examined for each category.

Recovery of hookworm larvae from pups' stomach

Two of the three pups selected for sampling had hookworm larvae in their stomachs. One animal (Pup A) had nine parasitic L_3 in its stomach sample on Day One and four larvae on Day Five. Pup A's faeces were positive for hookworm eggs at 30 days of age. For the other pup (Pup C), four parasitic L_3 were recovered from a stomach sample taken on Day Three but none at any later occasion; its faeces remained negative at 16 days of age. The larvae found in Pup C at three days old were longer than those observed in Pup A at both one and five days of age. Larvae found in Pup C on the fifth day had a provisional buccal capsule characteristic of fourth-stage larvae. None of the larvae had a sheath and measurements of body length are presented in Table 1.

Egg culture and hatching larvae

First-stage larvae (L_1) appeared within eggs after 15 days. The development of L_1, L_2, and L_3 took place within the egg-shell, with L_3 enclosed in the sheath of the previous stage. The eggs hatched naturally on the 23rd day and total length measurements of ensheathed free-living L_3 were then taken (n=11, 585.8 ± 15.5 µm). At this stage they only retained one sheath from the L_2 stage. On the 33rd day after the start of culture, the mean body length of 14 L_3 was 544.3 ± 31.0 µm.

Recovery of free-living stages and soil temperature

Figure 5.3 illustrates the sites where soil cores were sampled in 2003/2004 and in 2004/2005. Free-living third-stage hookworm larvae were extracted from one location on the beach on the 16th of January 2004 but not in December 2004, or January and February 2005. The other location where larvae were recovered was from sand adjacent to a small creek, on the 17th of February 2005. No free-living L_3 were recovered from mud or grass samples on the same date. The morphometric features of the larval specimens resembled those of free-living hookworm L_3 hatched *in vitro* (*cf. ante*). The diurnal temperatures at 20 cm deep on Sandy Bay beach from December 2004 to the end of January 2005 ranged between and 6.6 and 14.1º C and was significantly different from the temperatures measured in the field laboratory (range of temperatures for the same period: 3.7 to 22.9º C, $P<0.001$). The average amplitude of temperature near the egg cultures (7.75 ± 1.93º C) was much greater than in the sand by the creek (1.73 ± 0.12º C).

The life cycle of *Uncinaria* spp. in NZSLs is summarised in Fig. 5.4. It highlights the central role of pups as the definitive host and as the only source of contamination of the rookery with hookworm eggs.

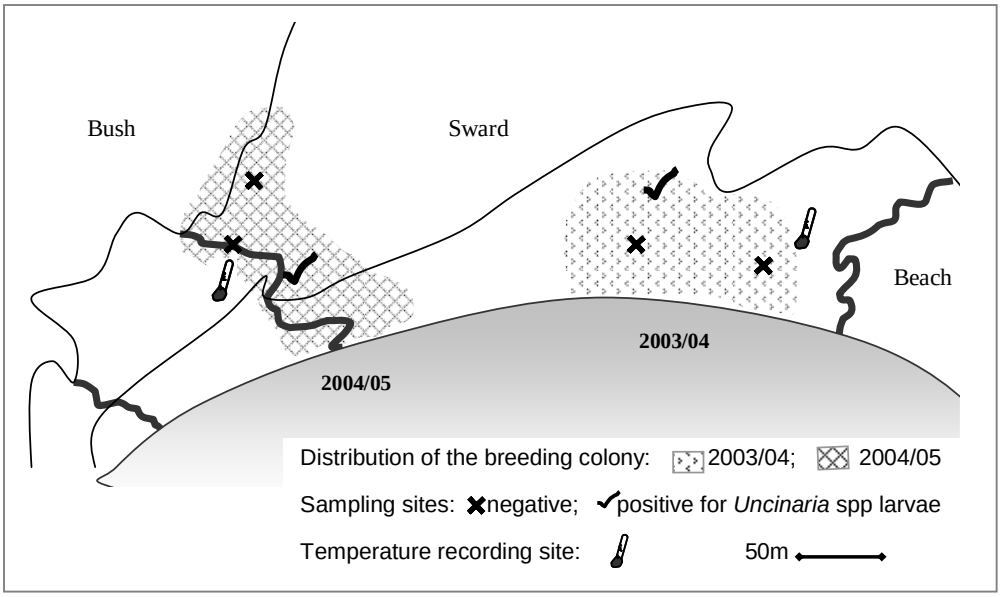


Figure 5.3 Sampling sites and location of temperature loggers on Sandy Bay Beach (Enderby Island). The beach is exclusively made of sand, the sward, of grass and the bush of small scrubs and trees. There are three creeks on Sandy Bay beach and pups spend most of the time swimming and playing in the muddy water. For both seasons sampling sites were located within the harem, as indicated by the two distinct patterns.

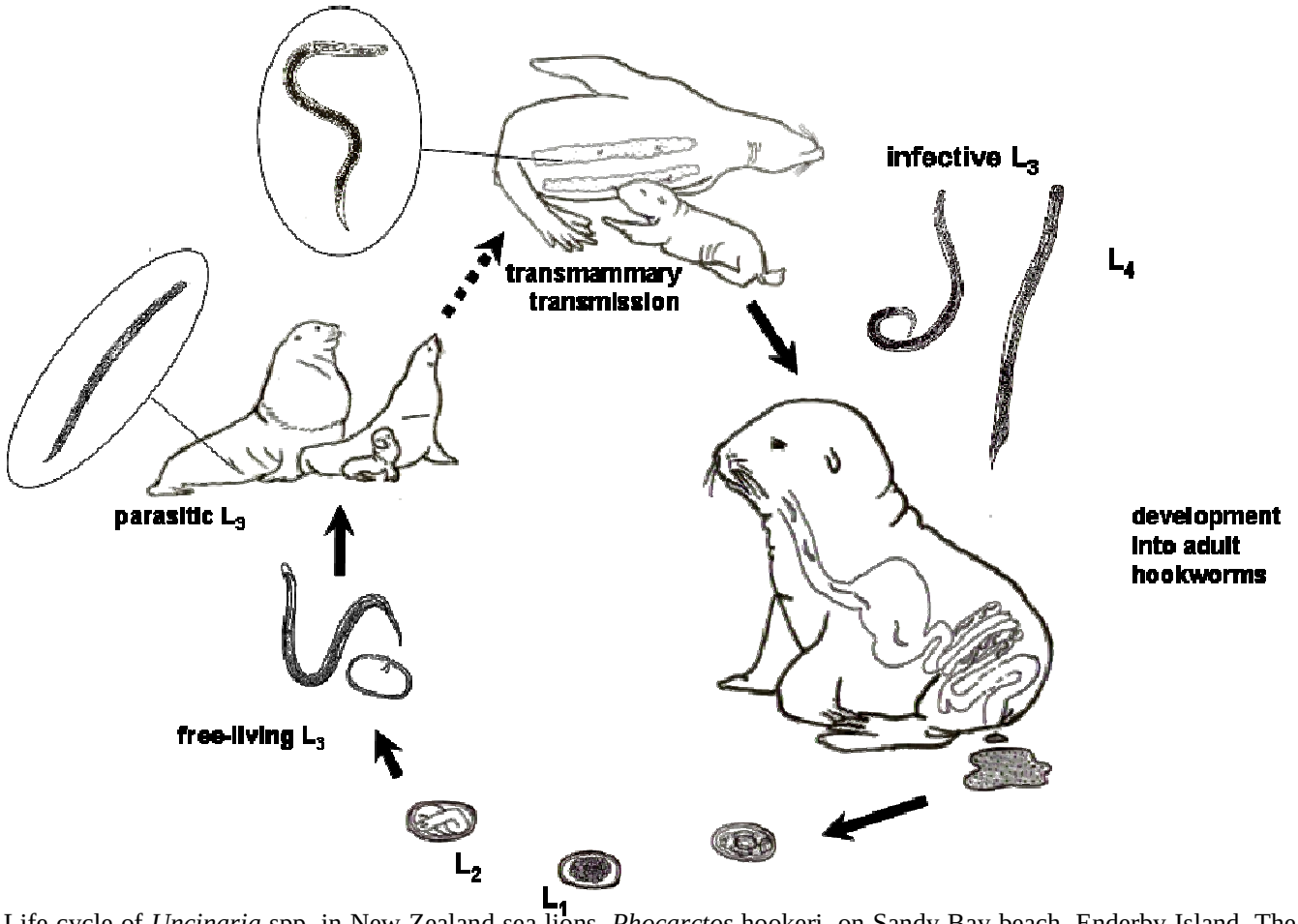


Figure 5.4 Life cycle of *Uncinaria* spp. in New Zealand sea lions, *Phocarctos* hookeri, on Sandy Bay beach, Enderby Island. The life cycle is based on data collected during the 2003/2004 and 2004/2005 breeding season studies on Sandy Bay beach.

This field investigation confirms that the life cycle of *Uncinaria* spp. in NZSLs is similar to that previously described for *Uncinaria lucasi* in northern fur seals (Lyons, 1963) and for *Uncinaria* spp. in California sea lions (Lyons et al., 2001) and in Juan Fernandez fur seals (*Arctocephalus philippi*) (Sepúlveda, 1998). A key feature of the life cycle of *Uncinaria* spp.. in all these species is that pups are infected only via ingestion of L_3 in the milk and during the first few days of life. Infections become mature after two to three weeks, meanwhile parasite eggs are deposited with pup faeces on the beach. These ova subsequently develop to ensheathed L_3 which seem to percutaneously infect all age groups and supply the L_3 that will infect future generations of pups.

Support for the role of transmammary infection in the present study comes from several different observations. Stomach contents of young pups contained L_3 on three of nine occasions in the first week of life. These were approximately the same size as those recovered from the mammary gland of a lactating female, supporting the transmammary transmission hypothesis. Further support can be seen in the age profile of the hookworms observed at necropsy. All hookworms appeared to be of a similar size and were apparently reaching patency at about the end of P1 indicating a highly synchronised time of infection. Sepúlveda and Alcaíno (1993), Lyons et al. (2000a) and Berón-Vera et al. (2004) observed a similar tight profile of developmental stage that they suggested was supportive of the hypothesis of transmammary infection. Another argument in favour of this hypothesis was that faeces of pups treated once with an anthelmintic drug at birth were negative until the end of the study. This raised the issue of anthelminthic persistence in NZSL pup tissues. Indeed, prolonged anthelmintic effects of the drug stored in the subcutaneous tissues and around the injection site could have masked any percutaneous infection of pups with free-living L_3 resulting in a patent infection. Arasu (1998) reported that ivermectin was the most effective anthelmintic drug in eliminating hookworm larvae in tissues of murine hosts, and Craven et al. (2002) observed that this highly lipophilic molecule was totally cleared from the fat of pigs between three and four weeks following a 300 µg/kg subcutaneous injection of ivermectin. In three NZSL pups treated with a single administration of ivermectin,

faecal analyses were still negative for *Uncinaria* spp. eggs, six weeks after the injection. Thus, in the present study, the absence of adult hookworms in the intestines of pups was probably not due to the residual activity of the anthelminthic drug, but rather to the early elimination of parasitic L_3 acquired via the colostrum.

Prenatal infection has been observed to play a minor role in the infection of terrestrial canine species of hookworm such as *Ancylostoma caninum* (Stone et al., 1970; Burke and Roberson, 1985). This issue has only been addressed once in otariids when no evidence was found that pre-partum infection occurred in northern fur seals (Lyons, 1963, 1994). Similarly, in the present study no larvae were found from seven placentas. However, this is not sufficient to definitively rule out any transfer of larvae prior to birth in NZSLs.

The prevalence of hookworm infection was very high on Sandy Bay Beach with up to 100% in some years, based on necropsy data. Transmammary transmission and prepatent period both contribute to explain the prevalence patterns observed each season on the rookery. In the NZSL, hookworm infection, as a direct cause of mortality and as a general diagnosis, was recorded more usually from mid-January (P2) every year, when pups were about three weeks old. This matches the hypothesis that P1, from birth to mid-January, corresponded to the prepatent period during which infective larvae were developing into mature adults in the pups' small intestine. However, development to the adult stage should have occurred earlier than the end of P1 as the first immature adult parasites of *Uncinaria lucasi* were observed after less than one week in northern fur seal pups (Lyons, 1963). Therefore, the difference between P1 and P2 should not have been so pronounced given that the prevalence at necropsy was based on the recovery of any parasitic forms, after sieving through a 250 µm-mesh and a 50 µm-mesh sieves. The cause for the failure to recover parasites during P1 was not apparent. For unknown reasons, the technical approach used in the present study to detect and count parasitic forms was not appropriate to retrieve immature hookworms. Despite efforts to manually scrape the mucosa whilst washing the intestines, hookworms were recovered on very few occasions and only near the end of P1. Immature stages may have been too deeply embedded in the mucosa to be successfully removed.

Hookworm infection as a primary cause of mortality has already been investigated by year in a general study on neonatal mortality in NZSL pups (Castinel et al., submitted *b*; *see* Chapter 2). Overall from 1998/1999 to 2004/2005 it was considered to be responsible for 13% of mortalities of pups at Sandy Bay Beach rookery. The present research showed that general prevalence of hookworm infection was lower in 2001/2002, which was the first year of the *Klebsiella pneumoniae* epidemics (Wilkinson et al., 2006), than in 2000/2001 and 2002/2003. The acute bacterial infections in 2001/2002 may have killed the pups before the development of infective larvae into adult hookworms was completed. Even though 2002/2003 was still seen as an epidemic season, there were less pups dying from bacterial infections as a primary diagnosis compared to the previous year (Castinel, submitted). An hypothesis could be that maternal antibodies from adults exposed to *K. pneumoniae* in 2001/2002 may have provided some measure of protection from the bacterium to pups born the following season.

Individual factors such as body condition and gender have been investigated for their influence on general prevalence, on hookworm infection as a direct cause of mortality and on the intensity of infection. In NZSL pups, body condition, as determined by the depth of their subcutaneous blubber layer, did not influence any of the three variables. This was in accordance with what is reported in South American sea lions regarding hookworm burdens (Berón-Vera et al., 2004) but differs from observations in Juan Fernandez fur seals (Sepúlveda, 1998), California sea lion (Lyons et al., 2005) and northern fur seal pups (Lyons et al., 2001). In the latter studies, pups in good body condition had more hookworms in their intestines than those in poor body condition. Assuming that transmammary transmission is the only way for pups to become infected and that hookworm burden is proportional to the quantity of milk ingested soon after birth, it is then reasonable to assume that pups in good body condition will have received more milk and therefore a greater amount of infective larvae (Lyons et al., 1997, 2001; Berón-Vera et al., 2004). Although there were significantly more males directly killed by hookworm infection than females, the burden in NZSL pups did not vary between sexes. Given that the relationship between numbers of hookworms and clinical disease is still not known in NZSLs, it would be difficult to speculate why male

pups, in good body condition, are more at risk of dying from hookworm infection than females.

Since the development of the two first larval stages were observed to take place inside the egg in the present study as well as in the study reported by Olsen and Lyons (1965), it seems very likely that free-living L_3 hatched near by the soil surface where the contaminated faeces were deposited. The developmental stages could therefore endure some large temperature variations. *Uncinaria* species seem to be adapted to a cool climate (Gibbs and Gibbs, 1959; Hill and Roberson, 1985) but it is unclear how long free-living hookworm larvae can survive in the soil. While Olsen and Lyons (1965) stated that some *U. lucasi* larvae had overwintered some years on Saint Paul Island, it did not occur with *Uncinaria* spp. on San Miguel Island, California (Lyons et al., 2001). At 20 cm beneath the sand surface, daily temperatures at Sandy Bay beach did not show much variation. The absence of great thermal amplitude may provide a temporary but clement environment for free-living hookworm larvae until they infect sea lions by the percutaneous route. However, the present study showed that hookworm larvae had not persisted in the sand in 2004/2005, at sites where these had been recovered in great numbers in February 2004. Survival of free-living larvae on Sandy Bay Beach may have also been compromised by the disappearance of almost all of the sand during the winter period, with only a narrow fringe of sand left on the very upper part of the beach. These observations could explain why free-living larvae would not persist on the rookery between the breeding seasons.

The presence of unsheathed parasitic third-stage larvae in the ventral abdominal tissues of all age categories strongly implicates that percutaneous and possibly oral infection with free-living L_3 occurred in NZSLs. The absence of larvae from the blubber of some adult females in 2004/2005 could have been the result of all or most of the larvae having migrated to the mammary glands and passed through the milk during early lactation. However, it was reported that adult female northern fur seals infected their pups with hookworms for their first pregnancy after being in captivity two to six years (Lyons and Bigg, 1983; Lyons and Keyes, 1984). This suggested that parasitic third-stage larvae could survive in tissues for several years. The blubber samples from the negative NZSL females were certainly too small to totally exclude the presence of

dormant larvae in the tissues. In addition, parasitic L_3 larvae were found in the blubber of NZSL adult sea lions in December 2004 when the soil samples were negative, indicating that infective hookworm larvae could survive for at least one year in the hosts' blubber. It was not possible to collect further data from NZSLs to determine the duration of survival of such larvae in tissues. The larvae found in mammary tissues of lactating females were of similar body length to those recovered from the stomach of pups. As described in northern fur seals (Olsen and Lyons, 1965), free-living third-stage larvae would penetrate sea lions through the skin, lose their sheath and stay in hypobiosis or arrested development in the blubber or abdominal muscles only; they would then migrate to the mammary glands in lactating females (Lyons, 1963). Lyons and Keyes (1978) hypothesized that a hormonal signal initiated the larval migration to the mammary cisterns but it has also been suggested that the decrease of immunocompetence, characteristic of preparturient females induced the larvae to resume their development and start their migration towards the glands (Anderson, 2000).

Pups given a single treatment of ivermectin did not get reinfected as indicated by negative faecal examination. Ivermectin may persist long enough to prevent all infection from the milk, while infective larvae were still being transmitted to pups. Nevertheless there were not enough data to determine whether the absence of reinfection in single treatment pups was due to the lasting effect of the anthelminthic dose or to the incapacity of percutaneously infecting larvae to develop into adult hookworms.

CONCLUSION – *Chapter 5*

Hookworm prevalence in New Zealand sea lion pups at necropsy was consistently high over the seasons, on Sandy Bay Beach. Transmammary transmission stands out as a key feature in the epidemiology of hookworms in NZSLs: it seems to be the exclusive route of infection to achieve a patent infection. It is likely that the vast majority of not all pups become infected by this route. Hookworm infection was associated with pup mortality but there was no evidence to relate parasite burden and clinical disease. Further investigations on all aspects of hookworm life cycle in NZSLs would be needed to reinforce the conclusions made from this first study and to elucidate when pups' intestines are cleared of hookworms.

Chapter 6

Clinical and pathological effects of hookworm (*Uncinaria* spp.) infection in New Zealand sea lion (*Phocarctos hookeri*) pups.

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ABSTRACT – *Chapter 6*

Hookworm infection with *Uncinaria* spp. in New Zealand sea lions (NZSLs) (*Phocarctos hookeri*) is an important cause of pup morbidity and mortality at Sandy Bay Beach (Enderby Island, Auckland Islands). As part of a health monitoring survey on this rookery, 455 pups were necropsied during the 1998/1999 to 2004/2005 breeding seasons and lesions associated with *Uncinaria* spp. were documented. In pups for which uncinariosis was diagnosed as the primary cause of mortality, the main gross lesion consisted of haemorrhagic enteritis characterized by frank blood and parasites in the intestinal lumen. When hookworm infection only contributed to death, mild enteritis, enlarged mesenteric lymph nodes, and fewer petechial haemorrhages were observed. For all pups, histopathological changes in the mucosa and submucosa mainly consisted of increased numbers of leukocytes especially in necrotic foci when bacteria were also observed. In addition, there was focal haemorrhage in Peyer's patches. In 2004/2005, the effect of hookworm infection on several haematological parameters was investigated by comparing "naturally infected" pups (n=24) with a "parasite-free" group (n=24) created by using an anthelmintic treatment. Blood samples were collected from 18 of these pups (controls and infected) and from 23 lactating adult females. Hematocrits (Hct) in neonates, less than five days of age (48.3%) and in adult females (50.0%), were similar ($P>0.05$), but Hct of pups between six days and about two months old were significantly lower (37.1%, $P<0.001$) than the two other categories. Hookworm infection had no significant influence on pup Hct ($P>0.05$) or on the other haematological parameters measured (total white blood cell (WBC), differential WBC and platelet counts, $P>0.05$). Overall the relationship between numbers of hookworms and clinical disease could not be clearly established from either the necropsy data or the comparative study in live pups.

The hookworms, *Ancylostoma* spp., *Necator* spp., *Bunostomum* spp., and *Uncinaria* spp. infect a vast range of mammalian hosts, including humans (Anderson, 2000). The clinical and pathological impact of these intestinal nematodes is highly variable between hosts and between parasite species (Prociv, 1997). For instance, *Ancylostoma caninum* is more pathogenic than *Uncinaria stenocephala* in dogs (Gibbs, 1958; Jubb et al., 1993) and than *Ancylostoma braziliense* in dogs and cats (Miller, 1966). Hookworm disease is characterized by iron-deficiency anaemia, malabsorption, and lethargy in dogs and humans (Pitchumoni and Floch, 1969; Tandon et al., 1969; Prociv, 1997; Hotez et al., 2004). Such pathological changes are largely the result of the feeding mechanisms of hookworms and the local mucosal trauma and bleeding they cause in the intestines (Garside et al., 2000). The buccal capsule of these parasites is armed with smooth cutting plates for *Uncinaria* spp. and teeth around the margin of the buccal capsule for *Ancylostoma* spp.. Both structures allow nematodes to burrow, anchor, and feed on the host's intestinal mucosa (Gibbs, 1958; Kalkofen, 1970; Loukas and Prociv, 2001). Primary bleeding is the direct consequence of submucosal capillaries bursting under the suction exerted by the parasite's buccal capsule. It is a discontinuous blood loss and therefore seems minimal compared to secondary bleeding due to trauma at the attachment site (Kalkofen, 1970). Haematological changes, associated with hookworm disease, involve the decrease of haemoglobin and erythrocyte concentrations (Dunbar et al., 1994). Decreased haematocrit (Hct) is a marker of anaemia (Kalkofen, 1987; Dunbar et al., 1994). In response to the presence of the parasite and to the local damage it causes, various inflammatory and haemostatic mechanisms are activated by the host (Jubb et al., 1993; Prociv, 1997) and by the hookworm itself with secretion of anticoagulant factors and digestive enzymes (Loukas and Prociv, 2001; Williamson et al., 2003). Haematological profiles of inflammation are very variable with the exception of eosinophilia, which has consistently been reported in neonates diagnosed with hookworm disease (Dunbar et al., 1994; Prociv, 1997; Tiwari et al., 2004).

In pinnipeds, *Uncinaria* species have been commonly reported in otariids (fur seals and sea lions) (Olsen, 1952; Botto and Mañe-Garzón, 1975; Sepúlveda and Alcaino, 1993; Lyons et al., 2000; Berón-Vera et al., 2004; Castinel et al., 2006) but there are

very few reports of these parasites in phocids (Dailey and Hill, 1970). Only two species, *Uncinaria lucasi* and *Uncinaria hamiltoni* have been described (Baylis, 1933, 1947), but intermediate types were reported (Olsen, 1958; Dailey and Hill, 1970; Castinel et al., 2006). Pups are the only age class to be affected by uncinariosis (Olsen and Lyons, 1965; Sepúlveda and Alcaino, 1993; Lyons et al., 2001). Hookworms have been associated with pup morbidity and mortality in several otariid species including northern fur seals (*Callorhinus ursinus*) (Keyes, 1965; Kato, 1997; Lyons et al., 2001), California sea lions (*Zalophus californianus*) (Lyons et al., 2001), and New Zealand sea lions (*Phocarctos hookeri*) (Castinel et al., submitted, *see* Chapters 2 and 5). Transmammary transmission has been described as the only mode of infection in otariids to achieve a patent infection (Olsen and Lyons, 1965). Yet, neonates do not necessarily succumb to hookworm infection. In studies on neonatal mortality in Juan Fernandez fur seal pups (*Arctocephalus philippi*) and in South American sea lion pups (*Otaria byronia*), there was no pathological changes observed at necropsy, despite the presence of hookworms (Sepúlveda and Alcaino, 1993; Berón-Vera et al., 2004, respectively). However, the intensity of infection in these two reports indicated the hookworm burden was less than 150 hookworms per individual, which is very low compared to that reported in other species for which the burden was often more than 1,000 hookworms per pup (Lyons et al., 2001; Castinel et al., submitted *a*, *see* Chapter 5). In northern fur seal pups with clinical uncinariosis, the principal necropsy findings consisted of haemorrhagic enteritis with resulting anaemia (Lucas, 1899; Olsen, 1958; Keyes, 1965). The histopathological changes described in California sea lion and northern fur seal pups included local infiltration of the intestinal mucosa by leukocytes and bacteria (Kato, 1997; Spraker et al., 2004). These histopathological lesions were consistently related to parasites which were anchored and feeding on the intestinal mucosa (Gibbs, 1958; Loukas and Prociv, 2001). Similar lesions have been noted in old feeding sites.

There have been only a few studies investigating the influence of hookworm infection on haematological parameters in otariid pups. Olsen (1958) reported lower values of Hct for northern fur seal pups with clinical signs of hookworm infection (range of 11-27%) compared to apparently healthy pups (range of 33-44%). However, the results were not consistent and some apparently healthy pups had a low Hct (22 and

23%). On San Miguel Island (California), there was a marked difference in the occurrence of hookworm disease between two otariid species sharing the same rookery (Lyons et al., 2001). In this study, northern fur seal pups, for which all faecal samples were negative for hookworm eggs, had a significantly higher Hct than California sea lion pups of the same age, which were almost all positive for hookworm infection (mean Hct of 42% and 30%, respectively). However, whether this difference was due to the parasite-host relationship or to interspecific variations between hosts is unknown.

The objective of the present study is to describe the post-mortem lesions directly associated with hookworm disease in NZSL neonates and to measure the short-term impact of *Uncinaria* spp. infection on NZSL pups' health using haematological parameters.

The present research is part of a larger study investigating the causes of neonatal mortality in NZSLs from 1998/1999 to 2004/2005 (Castinel et al., submitted *b*; *see* Chapter 2). Only data related to the pathogenicity of hookworms will be presented here. All NZSL pups (n=455) that died on Sandy Bay Beach rookery from early December to late February each year were necropsied using a standard protocol that examined all organs and systems. Samples were collected for histopathology, serology, parasitology, bacteriology and virology. Tissues and organs routinely sampled for histopathology included skin, skeletal muscle, brain, spinal cord, tongue, tonsil, thyroid, trachea, lungs, heart, lymph nodes, diaphragm, liver, spleen, pancreas, adrenal glands, kidney, stomach, intestine, urinary bladder, and gonads. Tissues were fixed in 10% buffered formalin and stained with haematoxylin-eosin (Luna, 1993).

Body condition of pups was rated in accordance with the abdominal blubber depth immediately posterior to the umbilicus (poor: less than 5 mm, moderate: 5 to 10 mm, fair to good: 10 to 15 mm, or excellent: more than 15 mm).

Total intestinal worm count was undertaken for 108 pups between 1998/1999 and 2004/2005. These were estimated by counts on 10% aliquots of total washings of the small intestine sieved through a 150µm-mesh sieve. In 2003/2004 and 2004/2005, faecal egg counts were estimated for ten necropsied pups and 14 live pups, using a modified McMaster technique where each egg counted represented 50 eggs per gram (epg). Full details on these parasitological techniques are presented elsewhere (Castinel et al., submitted *a*; *see* Chapter 5 and Appendices 5.4 and 5.5).

In 2004/2005, 48 pups born to known females were identified at birth by recording the number of the mother and by gluing a 100-mm plastic marker cap onto the pup's head and back (Chilvers et al., 2006a). They were paired by sex and date of birth. In order to create "parasite-free" pups to investigate the pathological role of hookworms, one pup from each pair was treated with ivermectin (200 µg/kg; Ivomec® injection for cattle, sheep and pig, 10g/L ivermectin, Merial New Zealand Ltd) at two days, one week, and one month of age. Two groups were formed: 1) the "parasite-free" pups,

treated with ivermectin, and 2) the untreated pups that were assumed to have acquired natural infections shortly after birth. This was confirmed by identification of hookworm eggs at about the end of the prepatent period (between 16 and 19 days of age; Castinel et al., submitted *a*; *see* Chapter 5). Pups were briefly held for less than 2 minutes and only when the mother was foraging at sea. Faecal samples were collected from each pup using a rectal loop approximately weekly or fortnightly to verify the efficacy of ivermectin and therefore the status of being "parasite-free" or that eggs were present in "naturally-infected" for each pup. Faecal analysis consisted of a direct smear if only a small amount of material could be recovered; otherwise, flotation of one gram of faeces with 28 ml of saturated NaCl solution was followed by microscopic examination (from x100 to x400 magnifications).

Blood samples were obtained from 18 different pups from both treatment groups (8 control and 10 infected pups) between birth and 58 days of age. Some individuals were sampled several times over the study period, resulting in a total of 45 samples. Blood was collected from the caudal gluteal vein, using 20 gauge x 38-mm needles, and stored in a tube with EDTA (Vacutainer®, Becton-Dickinson and Co., New Jersey, USA). Simultaneously, 23 lactating females, all mothers of marked pups, were captured for ongoing studies and blood sampling was conducted under anaesthesia. Haematocrit (Hct) values were obtained in duplicate by ultracentrifugation of heparinized microcapillary tubes, and by using a micro-haematocrit reader. Manual haematological techniques were employed in the field for total leucocyte and platelet counts using the Unopette system for white blood cells (WBC) (©Unopette, Becton-Dickinson and Co., New Jersey, USA). Blood smears were made and fixed in 100% ethanol in the field for May-Grünwald Giemsa staining in the laboratory. The proportion of segmented (mature) and band neutrophils, lymphocytes, monocytes, eosinophils, and basophils were estimated by differentiating 100 cells per slide.

In both pups and adult females, data were normally distributed for Hct, total WBC, platelet, segmented neutrophil, eosinophil and monocyte counts. Normality was obtained for lymphocyte counts after a square-root transformation. The correlation between pups' ages and Hct was tested using the Pearson's coefficient of correlation and with a scatter plot of Hct by age. Hct was tested for the effect of age (less than five

days-old or older) by analysis of variance (ANOVA). Hct values for each of the two pup groups were also compared to the group of adult females. The effect of hookworm infection ("parasite-free" Vs "naturally-infected" pups) on Hct, total WBC, segmented neutrophil, eosinophil, monocyte, square-root transformed-lymphocyte, and platelet counts was tested with a One-Way ANOVA. The analysis of variance was also used to compare haematological values between male and female pups. All statistical analyses of haematological parameters were performed using the statistical computer package SPSS (SPSS Version 12.0.1, ©SPSS Inc., IL, USA). Statistical analyses were not performed on epg counts due to the very small sample size. However, results are given as ranges followed by their median.

Hookworm burden and clinical disease

Post-mortem examinations conducted in 455 pups from 1998/1999 to 2004/2005 showed that hookworms were present in all pups with a mean burden of 824 parasites per individual (range: 50-8,329; 95% confidence interval for the mean burden: 136-3,323, Castinel et al., submitted, *see* Chapter 5). They were only found in the small intestines. Hookworm infection was a recurrent diagnosis, either as a direct cause of mortality (mean: 13%, range: 6-21%, Castinel et al., submitted, *see* Chapter 2) or as a factor contributing to mortality (range: 34%-64% depending on the year, Castinel et al., submitted *a*; *see* Chapter 5).

Faeces of necropsied pups found infected with hookworms were generally liquid, very dark and in some cases, stained with frank blood. The faecal egg count could be estimated on a limited number of pups during the 2003/2004 and 2004/2005 seasons. In dead pups (n=10), ranges of epg counts were [900-11,200] when uncinariosis was the primary cause of mortality (n=6 pups, median=6,450 epg) and [1,000-3,500] when it only contributed to death (n=4 pups, median=1,850 epg). For six of these necropsied pups, both faecal egg count and hookworm burden were available but no trend could be determined as the sample size was very small and neither the minimal nor the maximal burdens were associated respectively with the minimal and maximal egg count (*see* Appendix 6.1). Faecal egg counts in live pups were highly variable, ranging from 200 to more than 40,000 epg (n=14, median=7,000; *see* Appendix 6.1). The age of pups was generally not known. In three pups where the age was known, the faecal egg count was more than 40,000 epg at 30 days-old, 4,400 epg at 43 days-old and 8,000 epg at 50 days-old (*see* Appendix 6.1). No egg were seen in any faecal samples from the "parasite-free". Their faeces were still liquid but in contrast to all other infected pups, they were a light khaki colour.

Gross findings

Pups diagnosed with uncinariosis as the primary cause of death were in good (30%) to excellent body condition (60%), with a blubber layer consistently thicker than 10 mm. For pups infected with hookworms, but dying from another factor, body condition was extremely variable. In freshly dead pups, although not formally analysed, there was

no apparent relationship between pale buccal mucosal membranes and the presence of adult hookworms in the small intestines.

Internal examination of pups diagnosed with fatal uncinariosis as the primary post mortem diagnosis showed that lesions were confined to the intestinal mucosa and to the gut-associated lymphoid tissues (GALT). Pathological changes consisted of mild to severe haemorrhagic enteritis, with frank blood and adult parasites in the intestinal lumen. The intestinal serosa was generally pale but very dark bowel loops containing large amounts of frank blood in the intestinal lumen were also present. Scattered focal haemorrhages, corresponding to sites of attachment of hookworms, were observed on the mucosa and were visible from the serosal surface (Fig. 6.1a, 6.1b). Indeed, the gut wall was thickened in most cases but, in some fresh carcasses of pups with severe intestinal haemorrhage, the mucosa had become extremely thin, which gave the intestines a very dark and bloody tint. Peyer's patches and mesenteric lymph nodes were generally enlarged and haemorrhagic in some cases.

When hookworm infection was only considered to be a contributory cause of death, changes observed at necropsy were extremely variable and ranged from no visible lesions to mild haemorrhagic enteritis. In these cases, bacterial infections and trauma were generally the main causes of death.

Figure 6.1a Petechial haemorrage on duodenal serosa of a NZSL pup with hookworm enteritis. Bar=10mm

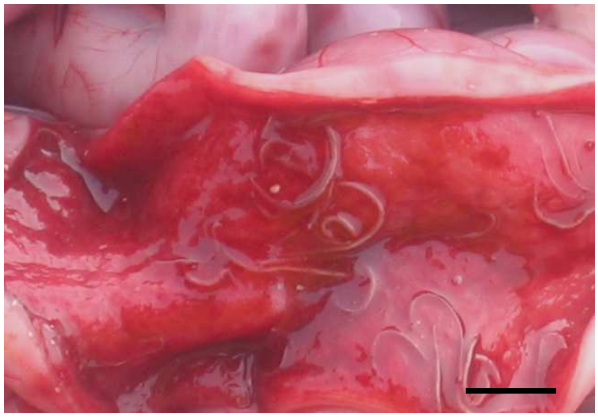

Figure 6.1b Adult hookworms (*Uncinaria* spp.) in the lumen of the duodenum in a NZSL pup with severe uncinariosis. Bar=5mm

Histopathology

All the histological changes directly associated with hookworm infections involved the intestinal tract, especially the duodenum, the mesenteric lymph nodes and the GALT.

Sections of small intestine frequently showed cross sections through hookworms which were both free in the lumen and embedded within the mucosa and submucosa (Fig. 6.2). Embedded nematodes were variably surrounded by a zone of necrosis or of congested tissue. These were both accompanied with infiltration by moderate numbers of neutrophils and plasma cells, with fewer lymphocytes and macrophages. No eosinophils were observed. These areas also contained numerous mixed colonies of bacteria. Vessels in the surrounding submucosa were generally markedly congested, with occasional haemorrhage. Similar areas of necrosis, containing bacteria and inflammatory cells, were often present in the mucosa in the absence of hookworms (Fig. 6.3a, 6.3b). In many sections of the intestine, there were moderate numbers of distended crypts containing degenerate inflammatory cells and necrotic debris. These areas of necrosis and distended crypts were probably previous feeding sites of hookworms.

The cortex of all mesenteric lymph nodes and the GALT contained increased numbers of plasma cells and neutrophils and focal haemorrhage was occasionally observed. Germinal centres were prominent.

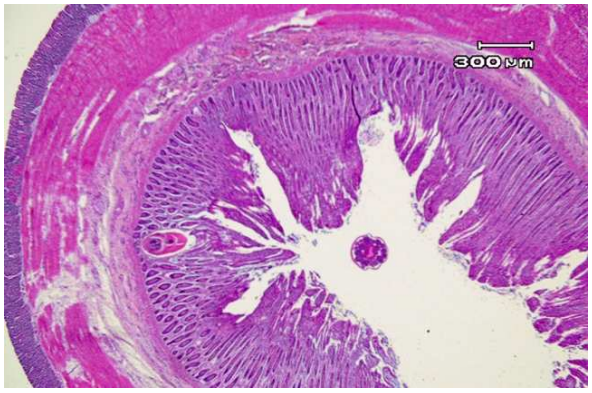

Figure 6.2 Transversal section of small intestine of a NZSL pup showing two sections of hookworms. There is no visible sign of villous atrophy or histopathological changes in the deeper layers.

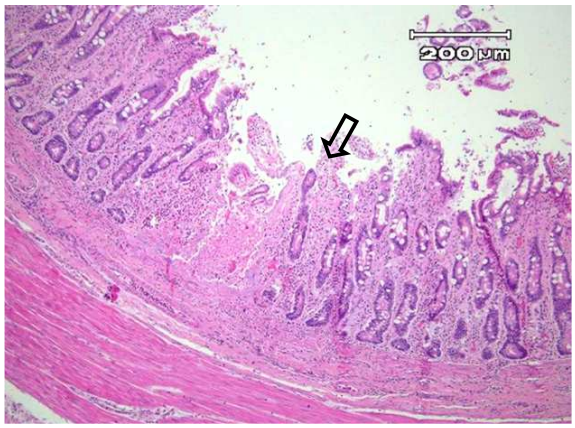

Figure 6.3a Histologic transversal section of duodenum of a NZSL pup with necrotic plug in a crypt (arrow). This corresponds to an old hookworm feeding site. Integrity of submucosa and muscles layers seem preserved.

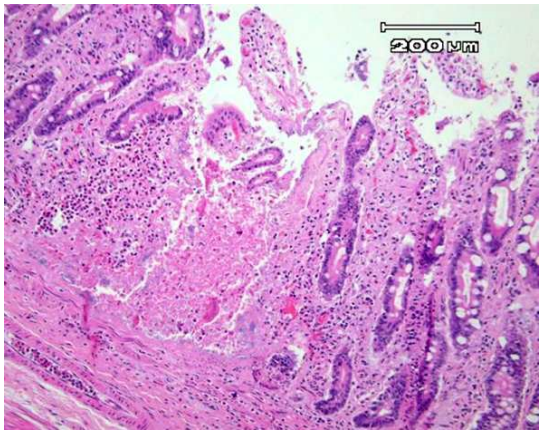

Figure 6.3b Higher magnification of Fig.6.3a, showing an old feeding site of hookworms in a NZSL pup.

Haematology

Haematocrit was measured to investigate clinical anaemia in live pups. Overall, there was no significant difference between "parasite-free" and "naturally-infected" pups or between male and female pups ($P>0.05$). Nevertheless, the scatter plot of Hct against pups' ages showed a dramatic drop in values five days after birth; then it continued a more steady decrease of Hct until about two months of age, which was at the end of the study period (Fig. 6.4). This breakpoint defined two age groups amongst pups. Hct values of newborns, from birth to five days old, were higher than for older pups ($P<0.001$), but did not significantly differ from adult females (Fig. 6.5). In contrast, Hct of pups between six days old and about two months of age was lower than the adult group ($P<0.001$). Mean Hct values for newborns and adult females were 48.3% and 50.0%, respectively, contrasting with 37.1% in older pups (Table 6.1). Total WBC counts were between 3,550 and 24,300 cells/μl in pups and between 3,450 and 42,400 cells/ μl in adult females (Table 6.1). Total WBC counts were not affected by pups' ages before or after 5 days of age, sex, or by the presence of hookworms ($P>0.05$). Even though WBC counts reached higher values in adult females, there was no significant difference between pups and females ($P>0.05$). Likewise, counts for all the different types of leucocytes overlapped between these two groups. Table 6.1 shows the mean percentages and ranges for each WBC subpopulation. The most abundant cell type was mature neutrophils, which represented 58.4% of the total leucocytes in pups and 57.2% in adults. Lymphocytes were the second most abundant WBC subpopulation (Table 6.1). No basophils could be identified on any blood smears. In addition, there was no influence attributable to the presence of hookworms, sex or age of the pups on any cell counts or platelet counts ($P>0.05$).

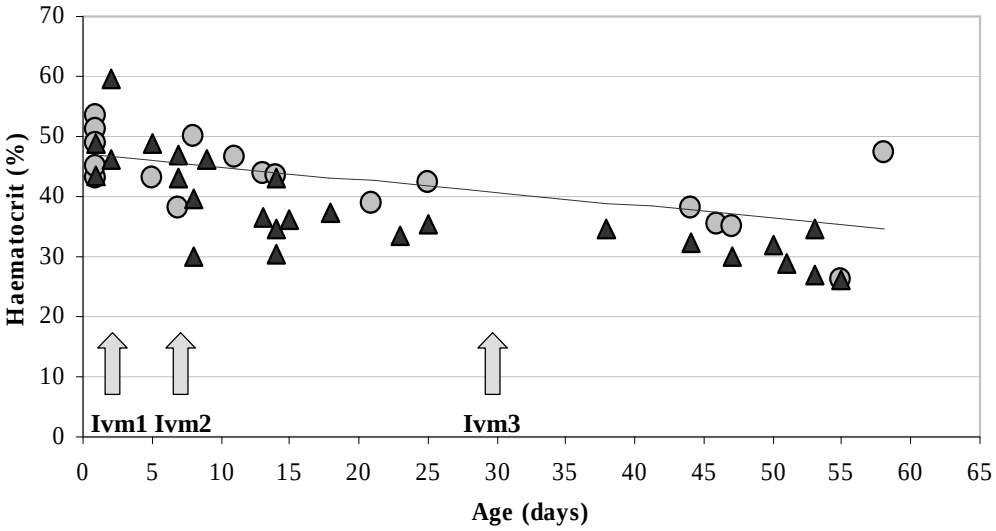

Figure 6.4 Haematocrit values of live NZSL pups by age and by treatment. Pups were either considered to be "parasite-free" following treatment with ivermectin (●) or remained "naturally-infected (▲). Tmes of treatment with ivermectin are indicated with bold arrows (Ivm1 at 2 days-old, Ivm2 at 7 days-old and Ivm3 at 1 month of age).

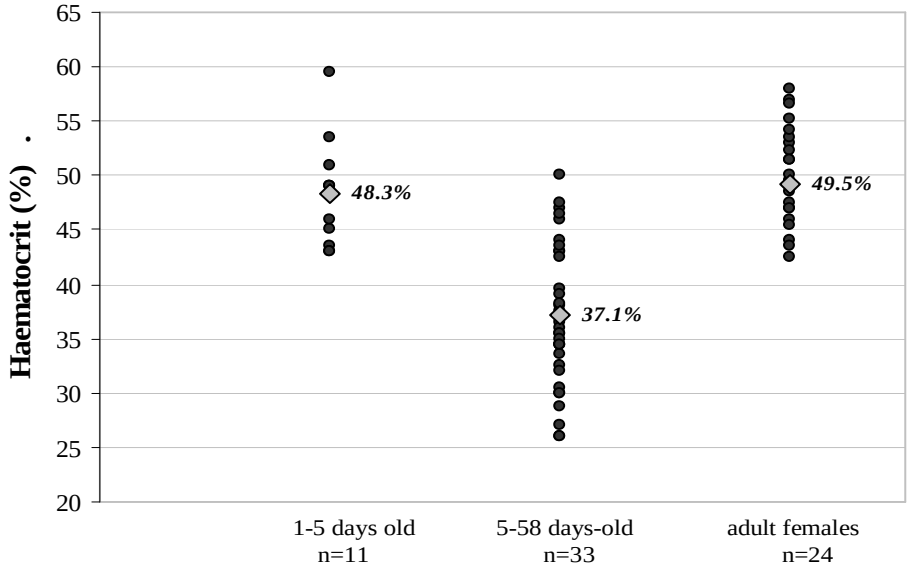

Figure 6.5 Individual haematocrit values (●) and group arithmetic means (◊) of NZSL pups and adult females. Pups were divided into two sub-categories (from 1 to 5 days of age and between 5 and 58 days of age).

	Pups (n=44)		Adult females (n=14)
Haematocrit (Hct) (%)	≤ 5 days-old	> 5 days-old	42-58 (50)
	43-59 (48)	26-50 (37)	
Platelets (10^3/mm^3)	0-976		47-438
Total WBC (/µl)	3,550-24,300 (8,995)		3,450-42,400 (12,154)
Band neutrophils (%)	0-972 (36)		0-1,700 (73)
Mature neutrophils (%)	1,065-20,169 (5,253)		1,483-33,150 (6,952)
Lymphocytes (%)	213-16,038 (2,321)		483-19,125 (3,488)
Monocytes (%)	71-9,720 (1,313)		103-12,325 (1,543)
Eosinophils (%)	0-1,458 (54)		0-8,075 (158)
Basophils (%)	0		0

Table 6.1 Range (mean) of haematological parameters measured in NZSL pups and adult females. Pups were divided into two groups (≤ 5 days-old and > 5 days-old). For lymphocyte counts, the back-transformed mean is given.

Post mortem findings associated with hookworm infection in NZSL pups were confined to the intestinal tract and, in most cases, consisted of haemorrhagic enteritis. However, after comparing blood parameters between "parasite-free" and "naturally-infected" pups, the presence of hookworms did not seem to influence haematological values of pups between birth and about two months of age.

Clinical and pathological manifestations of hookworm infection in mammalian hosts have been reported to be haemorrhagic enteritis leading to iron-deficiency anaemia as the major features (Pitchumoni and Floch, 1969; Miller, 1971; Tiwari et al., 2004) with other less important signs including skin lesions, diarrhoea, plasma protein loss, malabsorption, and more rarely secondary bacterial infections (Pitchumoni and Floch, 1969; Miller, 1971; Spraker et al., 2004; Tiwari et al., 2004). However, the term "hookworm disease" has often been misused in reports referring to morbidity of animals infected with hookworms (Miller, 1971). It is important to differentiate hookworm infection from disease. In this regard, unlike in some other otariid species from the Southern Hemisphere (Sepúlveda and Alcaino, 1993; Sepúlveda, 1998; Berón-Vera et al., 2004), the presence of adult hookworms in NZSL pups' intestines was generally associated with pathological changes and was determined to be the principal cause of mortality in 13% of the pups at Sandy Bay Beach rookery (Castinel et al., submitted *b*; *see* Chapter 2). In the most severe cases, the intestines had both thicker and thinner segments, similar to the changes described in northern fur seal pups by Lucas (1899) and Keyes (1965). Parasitic burdens in NZSL pups were generally over one thousand per individual (Castinel et al., submitted *a*; *see* Chapter 5), even when hookworm infection was not the primary cause of death. In comparison, the intensity of infection was reported to be lower in northern fur seal pups with fatal uncinariosis (mean of 760 hookworms per pup; Lyons et al., 2001). Therefore, it seems that most NZSL pups could tolerate greater numbers of parasites in their intestines before succumbing to the infection compared to northern fur seal pups, possibly because of their greater body size. The generally good body condition of pups with primary hookworm disease suggested that death had occurred very suddenly. It is likely that they could have died from acute anaemia following severe blood loss caused by high numbers of hookworms.

Assuming pups were infected in their first days of life through colostrum, parasites would have started feeding synchronously or at least in a very narrow time window. This acute phenomenon would explain why NZSL pups with uncinariosis as a primary cause of death were generally in good to excellent body condition. Based on the prepatent period following infection through the colostrum (Castinel et al., submitted a; *see* Chapter 5), pups diagnosed with hookworm infection as a primary cause of mortality would be about three-weeks of age. However, there were no data to support this hypothesis, since the age of pups could not be determined with accuracy at necropsy and none of the pups in the "naturally-infected" group died. Acute hookworm disease related to severe anaemia has been reported to be common in *Ancylostoma caninum* infections in canine pups (Miller, 1971). By contrast, *Uncinaria stenocephala* is not as pathogenic in dogs as *Ancylostoma* species: it has been suggested that the blood loss caused by *U. stenocephala* was lower compared to *A. caninum* (Miller, 1971; Kalkofen, 1987; Hotez et al., 2004). Uncinariosis in NZSLs appears to be more similar to ancylostomiosis in dogs.

Ivermectin has been reported to be effective in eliminating adult hookworms from northern fur seals (Beekman, 1984). However neither the duration of the drug efficacy nor its effect on tissue larvae acquired via percutaneous infection were investigated in that study. In the present study the absence of hookworm eggs in "parasite-free" pups indicates that this particular ivermectin treatment protocol was effective in removing adult parasites. Total WBC counts of NZSL pups could not be related to the presence or the absence of hookworms nor to any external signs of bacterial infections (e.g. conjunctivitis, suppurative wounds). There were no significant differences in differential WBC and platelet counts between "parasite-free" and "naturally-infected" pups and the absence of basophils noticed for both NZSL pups and adult females confirmed what had been previously reported for this species (Clark et al., 2002). Interestingly the eosinophil counts were low in both groups of pups. Eosinophil counts are commonly elevated in parasitised animals but there was no such evidence in this study.

Significantly lower Hct were found in pups of 5-58 days of age compared to newborns and adult females. This coincides with the period when maximal blood loss

would be occurring due to hookworms. However, there was no significant difference in Hct between “parasite-free” and “naturally-infected” pups. Although the number of samples and pups is small, it suggests there could also be a physiological explanation for the decline of Hct values observed in both groups after five days of age. General temporal variations of Hct values in these NZSL pups were similar to those reported in some other otariid species (Beckmen et al., 2003; Richmond et al., 2005). In free-ranging Steller’s sea lion pups, the lowest Hct was recorded around weaning time when they were three months old (Richmond et al., 2005). This was immediately followed by an increase of Hct and, by five months of age, values were similar to Steller’s sea lion adults. The augmentation of Hct could be the result of pups starting to dive at about five months to age to an increased depth, hence requiring higher haemoglobin concentrations to sustain the oxygen demand during such dives (Richmond et al., 2005). It seems that once pups start diving, their Hct increases. NZSL pups have been observed first going to sea at about one and a half months of age, but it is not known when repeated exposures to anoxia start to have a significant effect on their haematological values (Hawkey, 1975). Hct, like other blood parameters in pinniped neonates, have been reported to be influenced by their nutritional status (Rea et al., 1998) and their diving activity, which was demonstrated by studies comparing free-ranging and captive animals (Dierauf and Gulland, 2001; Lander et al., 2003). Erythrocyte size, haemoglobin concentration and iron levels were not measured in these NZSL pups, which made it impossible to determine if there was any evidence as to whether the anaemia was typically acute with normocytic and normochromic erythrocytes, or non-regenerative with microcytic and hypochromic erythrocytes as expected with chronic blood loss. Non-regenerative anaemia was reported in some northern fur seal pups infected with *Uncinaria lucasi* (Olsen, 1958). In dogs, old feeding sites of hookworms that had migrated to another feeding location have been observed to demonstrate chronic bleeding (Prociv and Loukas, 2001). Similar histological lesions were seen in NZSL pups but this observation alone did not supply sufficient evidence to confirm chronic blood loss leading to non-regenerative anaemia. In the present study, the only elements available for the diagnosis of anaemia were the visual examination of buccal mucosae in fresh carcasses at necropsy and Hct values. Pale carcasses were observed in pups diagnosed with hookworm infection regardless of whether it was a primary cause or it had only contributed to pup death. This suggested that visual assessment of

anaemia was not a reliable criterion. Adult females in this study had an Hct of 50%, which was within the range of values reported for NZSL adult females and juveniles by Costa et al. (1998).

Histopathological changes in NZSL pups with hookworm infection mainly consisted of infiltration of the intestinal mucosa and submucosa with moderate numbers of neutrophils, plasma cells and bacteria. Vessels surrounding hookworm feeding sites were occasionally observed to be markedly congested but the histological damage caused by *Uncinaria* spp. in NZSL pups was generally limited to the mucosa and submucosa. By contrast, hookworms have been observed to perforate the gut wall and cause peritonitis in California sea lion pups (Spraker et al., 2004). In that report, nematodes were observed to penetrate deeply into the mucosa, severely disrupting the epithelial integrity and some were even recovered in the peritoneal cavity of these pups. In the present study, although firmly anchored, hookworms did not extend beyond the submucosa and none were observed in the peritoneal cavity. As a result of the epithelial damage found in this study and reported by others, it is not surprising to find large numbers of bacteria in mesenteric lymph nodes, intestinal submucosa and muscularis. These bacteria may have entered through the disrupted mucosal barrier around the hookworm feeding sites (Garside et al., 2000). Combined with the presence of hookworms, the circulation of bacteria of intestinal origin results in a complex syndrome known as “hookworm infection with enteritis and bacteraemia” (HEB) (Spraker et al., 2004). However parasitic enteritis was only occasionally associated with septicaemia in NZSL pups at necropsy. The airways and the umbilicus are more likely routes of entry for bacteria causing septicaemia in pups but the possibility of hookworms compromising the integrity of the intestinal wall and leading to bacteraemia could not be ruled out.

Other lesions reported in animals with hookworm disease include skin lesions following percutaneous infection with larvae, diarrhoea and malabsorption syndrome (Miller, 1971; Hotez et al., 2004). Skin damage has been observed in cases of severe infection of dogs with *Ancylostama caninum* and *Uncinaria stenocephala*, often aggravated by self-mutilation triggered by local irritation and inflammation (Smith and Elliott, 1969; Miller, 1971; Hotez et al., 2004). Percutaneous infection also occurs in

NZSLs, regardless of their age (Castinel et al., submitted *a*; *see* Chapter 5) but such cutaneous lesions have never been seen in pups at necropsy or whilst alive. Determining if diarrhoea was present was difficult as all pups including those in the "parasite-free" group had liquid faeces and overtly watery faeces were never seen in any pups. Diarrhoea is another clinical manifestation of infection with hookworms but seems to be transitory and therefore less frequently reported than anaemia and skin lesions (Miller, 1971). However, black stools were frequently observed in NZSL pups at Sandy Bay Beach. At necropsy, very dark and blood-stained faeces were generally associated with heavy burdens of hookworms in the intestines (mostly over 2,000). Similar observations have been made in dogs and humans (Soulsby, 1982; Tiwari et al., 2004). Malabsorption of nutrients has been proposed as a significant issue in hookworm infection in several hosts (Kalkofen, 1970; Miller, 1971; Garside et al., 2000; Hotez et al., 2004) including northern fur seals (Brown et al., 1974) but in the present study, no attempt was made to measure nutrient uptake. With trichostrongylids in ruminants, the principle issue has been reported to be the loss of protein rather malabsorption per se (Coop and Holmes, 1996) and the situation with hookworms is probably similar. No differences in daily weight gain and weight at about two months of age were measured in NZSL pups treated with ivermectin (following the same protocol described in the present study) compared to naturally infected pups at Sandy Bay Beach (Chilvers et al., 2006a). These findings reinforced the hypothesis that hookworms did not affect growth in NZSL pups, from birth to about two months-old. Indeed, it seemed that only gender and weight at birth significantly contributed to variations of pup growth (Chilvers et al., 2006a).

Uncinariosis in otariid pups has not always been associated with mortality (Lyons et al., 2001; present study). In the present study, there appears to be a considerable variation in the pathogenicity of hookworm infection between pups. Some pups had very large faecal egg counts and appeared to tolerate the infection very well. The host-response may influence the pathogenicity of hookworms over and above direct factors such as blood loss. Indeed, regardless of differences in hookworm burdens, homozygosity for particular genes has been suggested to predispose California sea lion pups to anaemia (Acevedo-Whitehouse et al., 2006). Similarly, there may be genetic factors influencing the susceptibility of NZSLs to hookworm disease.

CONCLUSION – *Chapter 6*

The relationship between numbers of hookworms and clinical disease in NZSL pups could not clearly be established. Infection with *Uncinaria* spp. did not seem to invariably cause anaemia although intestinal lesions were generally observed. The haematological values presented here were collected during a breeding season without unusual mortality events and could serve as a reference point for future studies and for a more comprehensive survey of the NZSL pup population at Sandy Bay Beach, Auckland Islands. Further studies should be focussed on uncinariosis in older pups to determine the long-term impact of chronic infection and the time when they develop immunity to *Uncinaria* spp.

Chapter 7

Characterization of *Klebsiella pneumoniae* isolates from New Zealand sea lion (*Phocarctos hookeri*) pups during and after the epidemics on Enderby Island (Auckland Islands).

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ABSTRACT– *Chapter 7*

The 2001/2002 and 2002/2003 breeding seasons of New Zealand sea lions (NZSLs) on the Auckland Islands were marked by a high pup mortality caused by acute bacterial infections. As part of a health survey from 1998/1999 to 2004/2005, tissues and swabs of lesions had been collected at necropsy to identify the bacteria associated with pup mortality. *Klebsiella pneumoniae* was grown in pure culture from 83% of various organs and lesions in 2001/2002 and 76% in 2002/2003, and less frequently in the following seasons (56% in 2003/2004 and 49% in 2004/2005). Pup isolates of *K.pneumoniae* showed identical minimal inhibitory concentrations (MIC) of cefuroxime, neomycin, cephalotin, cephalexin and dihydrostreptomycin, suggesting clonal aetiology of the pathogen. Isolates also tested negative for production of extended-spectrum beta lactamases (ESBLs), which was not in favour of an anthropogenetic origin of the epidemic strain. Pulsed field gel electrophoresis (PFGE) of *Xba*I *DNA* macrorestriction fragments was performed on isolates of *K.pneumoniae* and *K.oxytoca* from 35 pups, the NZSL adult females, and from three human patients for comparison. PFGE showed that pup isolates of *K.pneumoniae* were genetically indistinguishable but were neither related to the *K.pneumoniae* isolates from humans and from NZSL adults nor to the *K.oxytoca* isolates from NZSLs. It is concluded that the 2001/2002 and 2002/2003 epidemics at Sandy Bay rookery were caused by a single *K. pneumoniae* clonal lineage , genetically different from the strain carried by adult NZSLs. There was no evidence that this *K. pneumoniae* clone was of anthropogenic origin, but further investigations are required to rule-out this possibility.

INTRODUCTION – *Chapter 7*

Klebsiella species are opportunistic bacteria, commonly found in the environment and in the intestinal flora of humans; they frequently cause nosocomial infections associated with high morbidity and sometimes mortality in hospitalized patients (Podschun and Ullman, 1998). In addition, Klebsiellae have been commonly cultured from assorted internal tissues in marine mammals (Vedros et al., 1982; Baker and McCann, 1989) and in a large range of wild birds, reptiles and terrestrial mammals (Aguirre et al., 1994; Montgomery et al., 2002; Steele et al., 2005). Nevertheless, *Klebsiella* species have never been associated with mass mortality in a wild animal population.

The New Zealand sea lion (NZSL) is one of the world's rarest and most highly localised pinnipeds. They were classified as "vulnerable" by the IUCN (Reijnders et al., 1993) and "threatened" under the New Zealand Marine Mammals Protection Act 1978 (Molloy and Davies, 1994). NZSLs breed on New Zealand's sub-Antarctic Islands between latitudes 48°S and 53°S (Gales and Mattlin, 1997). The 2001/2002 and 2002/2003 breeding seasons were marked by mass mortality of pups in the Auckland Islands breeding colonies (Wilkinson et al., 2006), with 31.3% of pup death in 2001/2002 and 21.7% in 2002/2003, compared to 10.9% in the previous year (Castinel et al., submitted *b*; *see* Chapter 2). Lesions of internal organs consistent with a bacterial infection were seen in a large number of carcasses and *K. pneumoniae* were subsequently isolated from the majority of these lesions.

Members of the family Enterobacteriacae, including *Klebsiella* species, can acquire plasmid-mediated extended-spectrum beta-lactamases (ESBLs) that provide resistance not only to penicillins but also to broad-spectrum cephalosporins, monobactams, and cephamycins (Hindler et al., 1994). The acquisition of such extended resistance is characteristic of Enterobacteriacae in the human environment and has become a key feature in the investigation of epidemic situations in hospitals (Gouby et al., 1994; Hindler et al., 1994). Pulsed-field gel electrophoresis (PFGE) of DNA macrorestriction fragments, a highly discriminatory whole-genome DNA-typing method for bacterial subtyping, is frequently used to characterize ESBLs-producing *Klebsiella pneumoniae*

strains involved in nosocomial infections and outbreaks (Gouby et al., 1994). PFGE has also been applied to the investigation of genetic relationships between strains of other Enterobacteriacae, such as *Escherichia coli* (Beutin et al., 2005) and *Salmonella enterica* (Dionisi et al., 2006).

This paper provides a microbiological and molecular characterization of *K. pneumoniae* isolates from NZSLs, associated with high pup mortality during the 2001/2002 and 2002/2003 breeding seasons on Sandy Bay Beach (Enderby Island).

Bacteriological analyses

From 1998/1999 to 2004/2005, all NZSL pups found dead on Sandy Bay Beach (Enderby Island, 50° 30'S, 166° 17'E) were necropsied as part of a health monitoring programme on the Auckland Islands. Lesions consistent with bacterial infections accounted for 24% of the primary causes of pup mortality during this period (Castinel et al., submitted *b*; *see* Chapter 2). During the two seasons of high pup mortality, in 2001/2002 and 2002/2003, the most common post mortem lesions were polyarthritis, peritonitis and meningoencephalitis, which were characterized by a collection of mucopurulent material in joints, peritoneal and encephalic cavities. Most lesions were acute, but chronic necrotising dermatitis was occasionally observed in a few pups at necropsy. Tissues and swabs from lesions were collected at post-mortem and stored in liquid nitrogen, until they were processed at Massey University (Palmerston North, New Zealand). The number of tissue samples analysed for bacteriology ranged from one to nine samples per pup. In addition, tissue samples from the carcass of one NZSL adult male from Otago Peninsula (South Island of New Zealand), and tissues from one adult female caught by a New Zealand Squid Fishery trawler and from two adult females found dead at Sandy Bay Beach were collected in 2004 and 2005.

At Massey University, tissue samples were cut open and material from the inside of the sample was inoculated onto sheep blood agar (SBA) and MacConkey plates using sterile swabs. Swab samples were directly inoculated onto plates. Plates were incubated in aerobic conditions at 37ºC for 24 to 48 hours. Subjective assessment of the bacterial growth was performed after the incubation period. If a mixed culture with no dominant colony-type was seen, the sample was considered contaminated. If a single colony-type or a mixed culture with a preponderance of one colony type was seen, one representative colony was picked for further analysis from either the MacConkey or the SBA. Gram-negative bacilli were identified phenotypically using Microbact^{TM} system test-kit (MedVet Science Pty. Ltd, Adelaide, Australia) according to the manufacturer's instructions. No other identification schemes were applied as no other bacteria were identified in the samples.

After the identification, bacterial isolates were stored in suspension in glycerol broth and frozen at minus 80 ºC for future reference.

Molecular characterisation with pulsed field gel electrophoresis (PFGE)

Forty *Klebsiella pneumoniae* isolates collected during the 2001/2002 and 2002/2003 breeding seasons and 25 isolates obtained in 2003/2004 and 2004/2005 were genotyped by pulsed field gel electrophoresis *(*PFGE) analysis of *Xba*I DNA macrorestriction fragments. Mostly, one isolate per animal was included in the analysis, but for four pups necropsied in 2004/2005, two isolates from different organs for each pup were tested to assess the similarity of isolates from the same animal. In addition, seven isolates of *Klebsiella oxytoca* and two *Klebsiella* spp. collected between 2001/2002 and 2004/2005 were included in the analysis. Three human isolates of *K. pneumoniae* (courtesy of Palmerston North Hospital microbiology laboratory), three isolates of *K. pneumoniae* grown from three adult female NZSL in 2004/2005 (one caught in New Zealand Squid Fishery trawl nets and two necropsied on Enderby Island), one *K. pneumoniae* isolate from the adult male necropsied on Otago Peninsula in 2004 and one *Klebsiella oxytoca* isolate from a NZSL pup in 2000/2001 before the mass mortality at Sandy Bay Beach were also analysed by PFGE. DNA extraction, digestion and PFGE conditions were largely as described previously by Fenwick et al (2004). Briefly, each NZSL isolate was grown overnight in brain-heart infusion broth, centrifuged, and resuspended in 50 microlitres of Pett IV buffer. The bacterial suspensions were mixed with 1% low melt agarose and transferred into plug moulds. The plugs were incubated overnight at 56ºC in Lysis buffer (0.5M EDTA [pH 8.0] + 1% sodium lauroyl sarcosine) containing 0.5 mg/ml of Proteinase K (Roche Diagnostics, Auckland, New Zealand). After lysis, a 2mm section was placed in restriction buffer containing 30 Units of the enzyme *Xba*I, and incubated at 37ºC overnight. The restriction fragments were separated by PFGE using the following run conditions: 6V per cm for 20 hours at a 120º angle, with an initial switch of 5 seconds and a final switch of 55 seconds. Lambda Ladder PFG and Low Range PFG Marker (New England Biolabs, Auckland, New Zealand) were included as molecular size standards. Gels were stained in ethidium bromide solution photographed under ultraviolet illumination. To infer phylogenies, a dendogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the pairwise distance matrix of Dice coefficient of similarity between the

DNA banding patterns, using the Diversity Database system (BioRad, Auckland, New Zealand).

Antimicrobial susceptibility and ESBL-testing

A twofold broth microdilution method was used to determine the minimal inhibitory concentration (MIC) of cephalexin (range tested: 0.125-16 μg/ml), cephalotin (range tested: 0.125-16 μg/ml), cefuroxime (range tested: 0.125-16 μg/ml), neomycin (range tested: 0.06-4 μg/ml) and dihydrostreptomycin (range tested: 0.06-4 μg/ml) against nine NZSL pup *K. pneumoniae* isolates using media, inoculum sizes, and a quality control strain (*Staphylococcus aureus* ATCC29213) in compliance with the National Committee of Clinical Laboratory Standards (NCCLS) recommendations (Anonymous, 2002). Six of these strains (three from 2001/2002 and three from 2002/2003) were tested for extended-spectrum beta-lactamase (ESBL) production using the disc diffusion confirmatory test recommended by NCCLS (Anonymous, 2002). Quality control strains for ESBL testing included *Escherichia coli* ATCC 25922- as negative control and *K. pneumoniae* ATCC 700603-strain as positive control.

RESULTS – *Chapter 7*

Over the period of study, 235 tissue and lesion samples collected at necropsy from pups at Sandy Bay Beach were cultured for bacteriology analyses. *Klebsiella oxytoca* was isolated in a single pup during the 2000/2001 season. All the non-lactose fermentor Gram-negative bacilli isolated from tissues between 2001/2002 and 2004/2005 were consistent with *Klebsiella* species using Microbact^{TM} and the vast majority (100 of 114 of these *Klebsiella* isolates) was identified as *K. pneumoniae* with more than 98% of confidence. Some degree of biochemical variability was observed between some of the isolates for tests of urease activity, Voges-Proskauer (VP) and arabinose fermentation; the other biochemical tests gave invariable results. Twelve isolates from six pups from 2003/2004 and 2004/2005 were associated with the *K. oxytoca* phenotype; two pups were infected with Gram negative rods, for which *Klebsiella* species could not be determined with more than 70% confidence.

Klebsiella pneumoniae was not isolated from NZSL pups that died during the 1999/2000 (n= 15 pups) and 2000/2001 (n = 4 pups) seasons, and was isolated for the first time during the 2001/2002 breeding season. In 2001/2002, this pathogen was grown in pure culture from multiple organs in 33 of 40 (83%) pups diagnosed with bacterial infection at necropsy and in 31 of 40 (76%) pups in 2002/2003. In the following seasons, the prevalence of *K. pneumoniae* isolated from pups at necropsy decreased to 56% (n=53 pups) and 49% (n=52 pups) in 2003/2004 and 2004/2005, respectively. *K.pneumoniae* was also identified in the adult male NZSL found dead on Otago peninsula, in the adult female NZSL caught in the New Zealand Squid Fishery trawl nets in 2004 and in the two adult females found dead on Sandy Bay Beach during the 2004/2005 breeding season.

PFGE analysis showed that the majority of pup isolates had an indistinguishable DNA banding pattern, but there were a few isolates with up to three bands difference (Fig. 7.1). The DNA banding patterns of two isolates from the same pup were invariably identical. The DNA banding patterns of *K. pneumoniae* from pups differed from the human isolates by 12 to 14 bands. One of the two unidentified *Klebsiella* species had the same banding pattern as the *K.pneumoniae* from pups, and the other was identical to

the *K.oxytoca* PFGE profile. The human isolates were different from each other and from all other *Klebsiella* species tested, by more than six bands. The DNA banding patterns of *K.pneumoniae* from adult females was different from each other and from the rest of the isolates but the *K.pneumoniae* profile of the adult male from the Otago peninsula was identical to that of pups from Sandy Bay Beach. The *K.oxytoca* isolates were different from each other and from *K.pneumoniae* from pups by more than six bands.

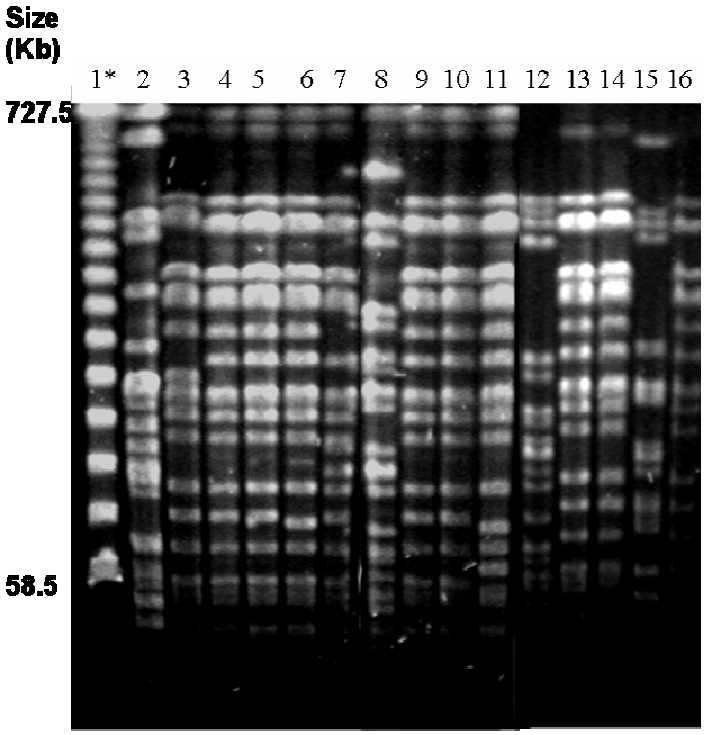

Figure 7.1 PFGE profiles of *Klebsiella* spp. isolates after restriction with *Xba*I. Lane 1* corresponds to a lambda ladder molecular marker; lanes 2, 8: human isolates of *K.pneumoniae*; lanes 3-7 and 9-11: *K.pneumoniae* from NZSL pups; lanes 12, 13, 15: *K.pneumoniae* from NZSL adult females; lane 14: *K.oxytoca*; lane 16: *Klebsiella* spp.

The UPGMA dendogram for *Klebsiella* species is shown in Fig. 7.2. The Dice coefficient of similarity between any pair of pup isolates was not lower than 0.85. Conversely, the Dice coefficients of similarity between *K. pneumoniae* from pups and from adult female NZSLs, or from humans, were lower than 0.5. This is depicted by a clear segregation between pup and non-pup isolates on the dendogram (Fig. 7.2).

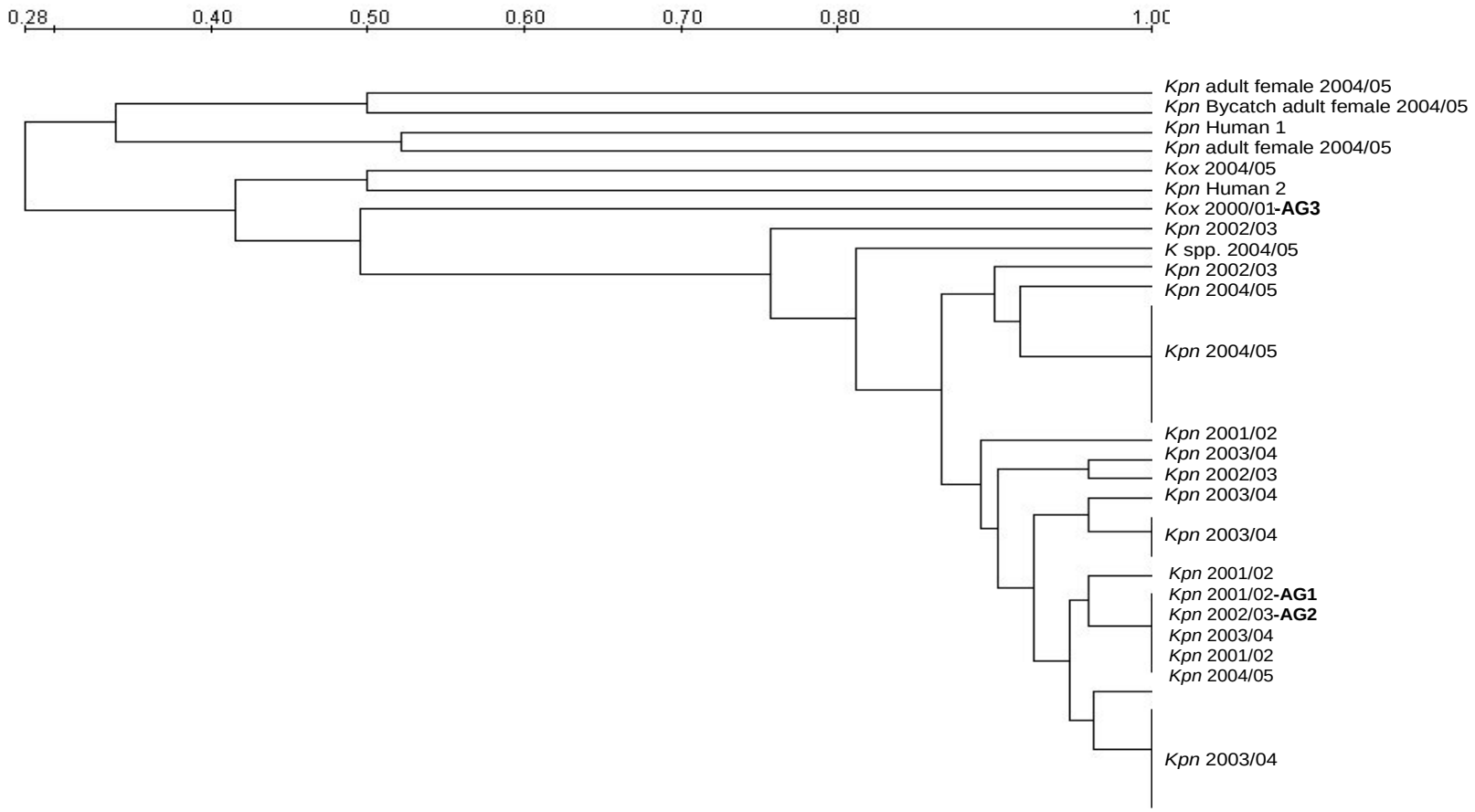

Figure 7.2 Similarity dendogram of 33 isolates of *Klebsiella* spp. from NZSL pups, 3 from adult females and two human isolates. The genetic parity between isolates is given as an index on the horizontal scale. AG1, AG2 and AG3 indicate the isolates used in the serological study in Chapter 9.

Likewise, the Dice similarity coefficients between *K. oxytoca* isolates and *K.pneumoniae* isolates from pups were lower than 0.5 (Fig. 7.2). Interestingly, two pup isolates identified as *K. oxytoca* with phenotypical tests showed an identical band profile as the *K.pneumoniae* isolates from pups.

There was only a low degree of variability in the MIC of the antimicrobials against the 12 pup isolates, with a maximum difference of two dilutions. MIC ranges were: cephalexin: 4-8 μg/ml; cephalotin: 2-4 μg/ml; cefuroxime: 2-8 μg/ml; neomycin: 2 μg/ml; dihydrostreptomycin: 2-4 μg/ml). None of the tested NZSL *Klebsiella isolates* produced ESBLs.

The 2001/2002 and 2002/2003 breeding seasons were characterised by high neonatal mortality on Sandy Bay Beach rookery, with an unusually high proportion of lesions consistent with bacterial infections at necropsy. *Klebsiella pneumoniae* was identified as the major causative agent of the two-year mass mortality. There were several bands of difference between isolates of *K. pneumoniae* from pups and those from adult females in 2004/2005. Interestingly, isolates of *K.pneumoniae* obtained from adult females in 2004/2005 at Sandy Bay Beach were not related to those from pups, for the same season and at the same site. Interestingly the isolate from the adult male found dead on Otago peninsula in 2004was identical to the pup strain.

Klebsiella oxytoca was found in a single pup necropsied on Sandy Bay Beach in 2000/2001, with only moderate bacteria-induced pathological signs at post-mortem. The high rate of acute, severe bacterial lesions observed in pups in 2001/2002 and 2002/2003, suggested an epidemic with a highly pathogenic *Klebsiella* strain on the rookery. Highly invasive *K.pneumoniae* are emerging as causes of internal organ abscesses in humans, and the genetic makeup of these pathogens is currently under study (Ma et al., 2005). The genetic similarity between pup isolates of *K. pneumoniae* from 2001/2002 to 2004/2005 strongly suggests the emergence and persistence of a highly pathogenic clonal lineage in the NZSL population over four breeding seasons.

The clonal hypothesis for *K.pneumoniae* isolated from NZSL pups at Sandy bay Beach in 2001/2002 and 2002/2003 and during the following seasons was confirmed by the PFGE analysis, and by the extremely low MIC and biochemical variability. These isolates formed a large distinct cluster, with over 90% of them presenting similarity coefficients greater than 0.75. The presence of two *K.oxytoca* phenotypes within this group of genetically closely related isolates underlines the fact that phenotypic identification may not be as accurate as molecular sub-typing like PFGE to characterise *Klebsiella* species in an epidemic situation.

Such clonal lineage could have originated from different sources: it could have emerged from pre-existing strains by acquisition of virulence factors via horizontal gene

transfer, as suggested by a cluster of *K.pneumoniae* causing liver abscess in humans (Lau et al., 2000). Likewise, the introduction of a new, highly pathogenic clonal lineage possibly from human origin could have caused the mass mortality events in a naïve wild population. Since small numbers of adult male and juvenile NZSLs are found on the Otago Peninsula, which is close to the populated city of Dunedin (South Island of New Zealand), it is conceivable that these animals had been infected with human Klebsiellae from sewage contaminated waters. The animals could then serve as vectors between the New Zealand South Island and the breeding colonies on the Auckland Islands, as suggested by the presence of the pup clonal epidemic strain in an adult sea lion on Otago peninsula in 2004. This adult was probably infected at Sandy Bay Beach rookery and travelled to the New Zealand mainland. A reverse transfer is just as likely. Human strains of *Klebsiella* might be expected to show increased resistance to antimicrobial agents (Briggs et al., 2005). Hence, ESBL testing of NZSL isolates was undertaken to possibly obtain an epidemiological clue for an anthropogenic origin of the clone, as ESBL-positive strains have been identified in the human environment. Reports on antimicrobial susceptibility profiles of *K.pneumoniae* in New Zealand are scarce (Karalus et al 1991; Briggs et al 2005), but *Klebsiella* ESBL-positive strains are also present in New Zealand (http://www.surv.esr.cri.nz/PDF _surveillance /Antimicrobial/ ESBL_2005.pdf). All NZSL pup isolates tested were negative for ESBL production, and given the evidence for monoclonal origin of the isolates, such a result can be generalised to the entire number of isolates. Yet, the absence of ESBL production by itself is not sufficient to rule-out an anthropogenic origin of the epidemic *K. pneumoniae.*

CONCLUSION – *Chapter 7*

In conclusion, results of this molecular and phenotypic investigation indicate the emergence of a single highly pathogenic *K. pneumoniae* clonal lineage that caused mass mortality in NZSL pups during two consecutive breeding seasons on the Auckland Islands. No anthropogenic source of infection could be established. More research on the genetic makeup of the isolates would be needed to elucidate the circumstances surrounding the emergence of such a highly pathogenic clone on the rookery.

Chapter 8

Description of the 2002 and 2003 *Klebsiella pneumoniae* epidemics in New Zealand sea lions on Enderby Island (Auckland Islands).

To be submitted for publication
to the *Journal of Wildlife Diseases*

ABSTRACT – *Chapter 8*

The 2001/2002 and 2002/2003 breeding seasons in the New Zealand sea lion (NZSL) were marked by unusually high pup mortalities at Sandy Bay Beach rookery (Enderby Island, 50° 30'S, 166° 17'E), with a dramatic increase in deaths due to bacterial infections. A variety of suppurative lesions were observed at necropsy, with arthritis of one or more joints being the most frequent post-mortem finding during these two epidemic seasons. Other consistent lesions were cellulitis, peritonitis and meningitis. *Klebsiella pneumoniae* was constantly isolated from affected tissues. Gross and histopathological findings from pups necropsied between 2001/2002 and 2004/2005 at Sandy Bay Beach are presented. Internal lesions were consistent with septicaemia, which explained the wide range of organs from which the pathogen was grown in pure culture.

INTRODUCTION – *Chapter 8*

Bacterial infections often cause or contribute to death of marine mammals (Stroud and Roffe, 1979; Gulland et al., 1996; Thornton et al., 1998). Bacteria previously reported to cause severe lesions and/or fatal septicaemia in pinniped species include salmonellosis in northern fur seals (*Callorhinus ursinus*) and northern elephant seals (*Mirounga angustirostris*) (Stroud and Roelke, 1980; Vedros et al., 1982; Stoddard et al., 2005), leptospirosis in California sea lions (*Zalophus californianus*) (Gulland et al., 1996; Acevedo-Whitehouse et al., 2003a), and tuberculosis in Australian fur seals (*Arctocephalus pusillus*) (Cousins et al., 1993; Woods et al., 1995).

It is also common to isolate commensal micro-organisms from various tissues of pinnipeds at necropsy without any associated lesions (Vedros et al., 1982; Foster et al., 2002; Hernandez-Castro et al., 2005). Opportunistic bacteria such as *Streptococcus* spp., *Staphylococcus* spp., *Escherichia coli, Klebsiella* spp. and *Proteus* spp. have been commonly isolated in free-living pinnipeds (Stroud and Roffe, 1979; Vedros et al., 1982; Johnson et al., 1998; Thornton et al., 1998).

Klebsiella species are ubiquitous bacteria found in both marine and terrestrial environments (*see* Chapter 1, Section IV.4.1). They infect a wide range of animals and have been reported to commonly cause nosocomial infections (Podschun and Ullman, 1998).

During the 2001/2002 and 2002/2003 breeding seasons of NZSLs, *Klebsiella pneumoniae* was identified as the causal agent of bacterial epidemics at Sandy Bay Beach rookery (Castinel et al., submitted *b*; *see* Chapter 2). Both seasons were characterized by high pup mortality, respectively 31.3% and 22.1%, compared to 10.2% in the non-epidemic years (Castinel et al., submitted *c*; *see* Chapter 7).

This chapter will describe some aspects of the epidemiology of the *Klebsiella pneumoniae* epidemics during two consecutive breeding seasons at Sandy Bay Beach rookery on the Auckland Islands compared to previous years and to those following; provide a description of gross and histopathological lesions observed in pups; and comment on the possible pathogenesis of infection by this bacterium as a cause of mass mortality in the vulnerable NZSL.

MATERIALS AND METHODS – *Chapter 8*

Full necropsy of every NZSL pup found dead on the rookery was conducted as part of a health monitoring programme run at Sandy Bay Beach (Enderby Island, 50° 30'S, 166° 17'E, Auckland Islands) from early December to mid-February for every austral summer. The data analysed here cover the period from 1998/1999 to 2004/2005. During these seven consecutive years, 108 pups were necropsied before the *Klebsiella pneumoniae* epidemics, 234 during the epidemics and 113 in the following seasons. Primary causes of neonatal mortality were categorised as stillborn, trauma, starvation, bacterial infection and hookworm enteritis (Castinel et al., submitted b; *see* Chapter 2).

A range of tissues were collected at post-mortem for bacteriological analyses. Tissues routinely sampled included liver, lung, tonsil, thymus, lymph nodes, spleen, faeces and any lesions or exudates; these were stored in liquid nitrogen.

Samples of skeletal muscle, brain, spinal cord, tongue, tonsil, thyroid, trachea, lungs, heart, lymph nodes, diaphragm, liver, spleen, pancreas, adrenal glands, kidney, stomach, intestine, urinary bladder, and gonad were collected for histopathology and fixed in 10% buffered formalin. Formalin fixed tissues were processed routinely at Massey University (Palmerston North, New Zealand) for histopathology. Sections were stained with haematoxylin and eosin and examined microscopically. In the present study, details of observations are given for six pups from the 2001/2002 and 2002/2003 breeding seasons and ten pups from the 2003/2004 and 2004/2005 seasons.

Techniques of culture and phenotypic identification of bacterial isolates as well as molecular characterization of the strain of *Klebsiella pneumoniae* were described in Chapter 7. Prevalence and nature of bacterial lesions associated with this pathogen over the survey period are presented and discussed in the present research.

RESULTS – *Chapter 8*

Bacteriology results are reported in more detail in Chapter 2. Briefly, a clonal strain of *Klebsiella pneumoniae* was isolated from pups at necropsy in 2001/2002 for the first time and for every subsequent breeding season until the end of the period of survey in 2004/2005. This pathogen was grown in pure culture from tissues of a large majority of dead pups diagnosed with bacterial infections during the 2001/2002 and 2002/2003 epidemics at Sandy Bay Beach (83% and 76% respectively, *see* Chapter 7).

The general prevalence of bacterial infections at post-mortem and their proportion as a primary cause of death are presented in Figure 8.1. General prevalence of bacterial diseases increased from 23.0% before 2001/2002 to 68.7% during the epidemics then decreased to 35.5% in the following seasons (Fig. 8.1). However the ratio of bacterial infection as a primary cause of death to the general prevalence of bacterial infection did not show great temporal variation over the period of study: approximately 0.6 before the epidemic seasons, 0.5 during the epidemics and 0.4 post-epidemics. Prevalence of *K. pneumoniae* isolated from pups diagnosed with bacterial infection decreased after 2001/2002 and 2002/2003, with 56% in 2003/2004 and 49% in 2004/2005 (*see* Chapter 7). The daily incidence of bacterial infections is illustrated from 2000/2001 to 2003/2004 (Fig. 8.2). Bacterial infections recognised as a primary cause of mortality were generally observed for the first time between the 25th to 35th days of the season, when pups were about two weeks of age. They represented a high proportion of daily pup mortality until the end of the season, between the 71st and the 82nd days (Fig. 8.2). In 2000/2001 (“2001” on Fig. 8.2), bacterial infections as a direct cause of death, accounted for less than 20% for the last third of the season. In contrast, these were associated with over 50% of daily pup mortality from the 60th day (approximately the end of January) until the end of the season, during the epidemics. In 2003/2004 (“2004” on Fig. 8.2), the proportion of bacterial infections returned to values similar to these prior to the epidemics.

A wide range of macroscopic lesions were found in various organs in pups diagnosed with bacterial infections at necropsy in 2001/2002 and 2002/2003 (Table 8.1). A characteristic macroscopic feature seen at sites of inflammation was the presence of

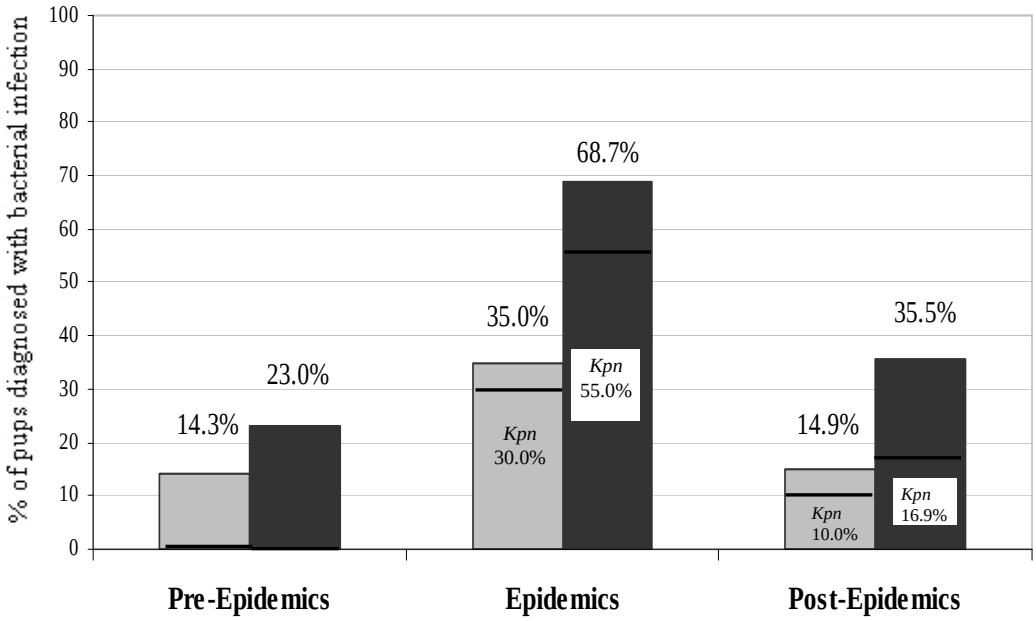

Figure 8.1 Bacterial infection diagnosed as the primary cause of death (light grey) in NZSL pups and presence of lesions due to bacterial infection (dark grey). The proportion of pups with *Klebsiella pneumoniae* (*Kpn*) within those diagnosed with bacterial infection is indicated underneath *Kpn*. No bacterial isolates were identified as *K. pneumoniae* before the epidemics.

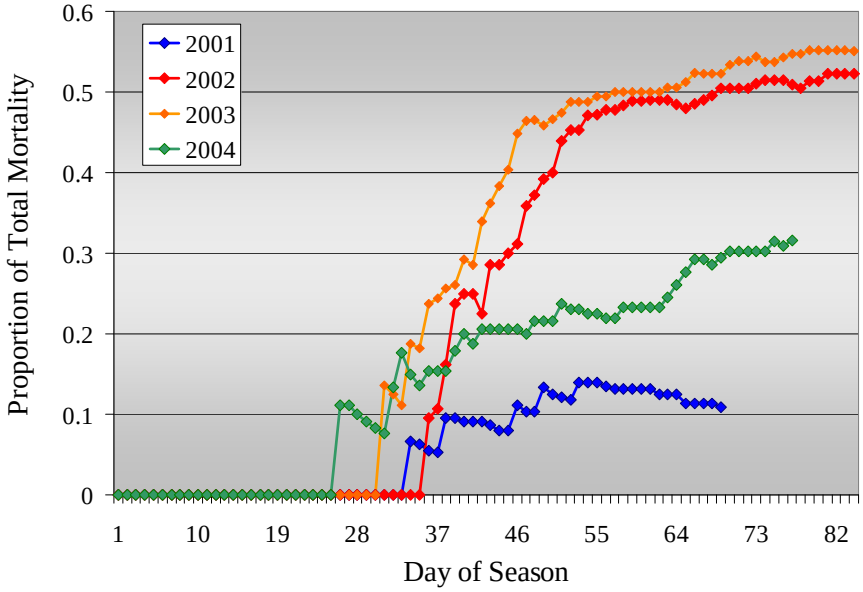

Figure 8.2 Incidence of bacterial infection as a proportion of daily pup mortality before (2000/2001 or "2001"), during (2001/2002 and 2002/2003, respectively "2002" and "2003") and after (2003/2004 or "2004") the *Klebsiella pneumoniae* epidemics at Sandy Bay Beach. Bacterial infection was associated with over 50% of pup mortality in 2002 and 2003.

	2001/2002	2002/2003
No. of pups diagnosed with bacterial infection(s)	**50**	**54**
Monoarthritis	10 (20%)	35 (65%)
Polyarthritis	15 (30%)	9 (20%)
Cellulitis/abscess	16 (32%)	7 (13%)
Peritonitis	10 (20%)	16 (30%)
Bronchitis	3 (6%)	5 (9%)
Pneumonia	3 (6%)	5 (9%)
Meningitis	3 (6%)	11 (20%)
Necrotic dermatitis	2 (4%)	0
Pyothorax	1 (2%)	1 (2%)
Vaginitis	1 (2%)	0
Enteritis (bacterial)	0	2 (4%)

Table 8.1 Most common post-mortem lesions caused by bacterial infection in New Zealand sea lion pups, on Sandy Bay Beach (Enderby Island), for the 2001/2002 and 2002/2003 seasons. Percentages do not add up to 100% since bacterial infection was often diagnosed in multiple organs for each pup.

abundant thick purulent exudate (Fig. 8.3 and 8.4). The most common gross findings in January and February 2002 (2001/2002 season) were acute arthritis and cellulitis (Table 8.1). Carpal and tarsal joints were a regular site of infection (Fig. 8.3) and some purulent material was often found in peripheral tendon sheaths and surrounding soft tissues (Fig. 8.4). In addition, bacterial infection could affect two or three adjacent joints of the same limb (polyarthritis). Monoarthritis and polyarthritis were diagnosed respectively in 20% and 30% for the 2001/2002 breeding season and in 65% and 20% in 2002/2003 (Table 8.1). The frequency of arthritis in general was higher in 2002/2003 than for the previous year (44 out of 54 pups compared to 25 out of 50 pups in 2001/2002). Suppurative arthritis of the atlanto-occipital joint was reported in ten pups during the epidemics but not afterwards. Meningitis and peritonitis were more frequently observed at necropsy during the second epidemic season (respectively 20%

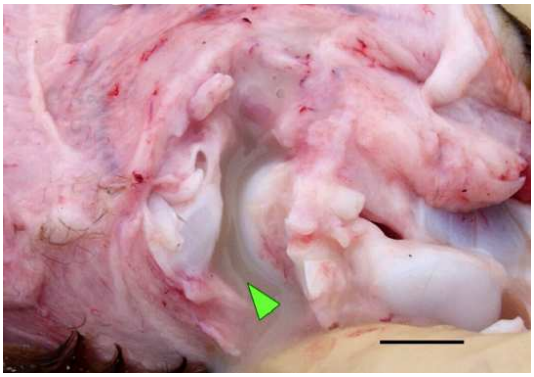

Figure 8.3 Suppurative exsudate (arrow) within tarsal joint space of a NZSL pup during the *Klebsiella pneumoniae* epidemics. Bar = 1cm.

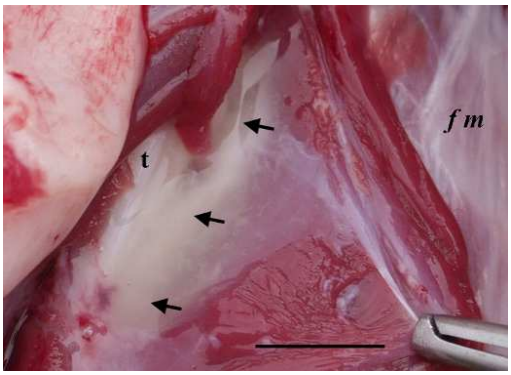

Figure 8.4 Suppurative tenosynovitis and cellulitis surrounding the carpal joint of a NZSL pup during the epidemics. Fascia muscularis is indicated by *fm* and tendon by *t*.Bar=2cm.

and 30% in 2002/2003 compared to 6% and 20% in 2001/2002). Necrotic dermatitis of the face and head was diagnosed in two pups in 2001/2002 while bacterial enteritis and septicaemia were only reported in the following season (Table 8.1). Gross diagnosis of septicaemia was supported by generalization of bacterial infection with purulent exudate to organs of both thoracic and peritoneal cavity and to several joints.

Histopathological findings in six pups necropsied during the 2001/2002 and 2002/2003 breeding seasons and in ten pups during 2003/2004 and 2004/2005 are listed in Table 8.2. All major organs and systems showed microscopic changes, whether bacterial infections were the primary or a contributory cause of death. Sites of inflammation included the respiratory tract, central nervous system, myocardium, lymphoid system, liver and alimentary tract. The diffuse pulmonary congestion seen in four of six pups from the epidemic period and in half of the pups in the following seasons (Table 8.2) seems to be a non specific change frequently observed at post mortem, rather than a specific lesion induced by *K. pneumoniae*. The generalised lymphoid depletion suggested a systemic illness, and reactive lymph nodes indicated the presence of inflammation in the region they drained. Lesions of the central nervous system (such as sub-arachnoid haemorrhage, meningitis and meningoencephalitis) were only seen in 2001/2002 and 2002/2003. Sections of brain from pups necropsied during the 2001/2002 epidemic season showed expansion of the meninges by oedema fluid and

	Epidemic seasons No. of pups with		Post-epidemic seasons No. of pups with	
	primary lesions	secondary lesions	primary lesions	Secondary lesions
No. of pups examined	3	3	6	4
RESPIRATORY SYSTEM				
Diffuse congestion	3	1	6	3
Interlobular oedema	1	.	2	.
Alveolar atelectasis	.	1	.	.
Alveolar emphysema	.	.	.	1
Acute suppurative pleuritis	.	.	.	1
Pneumonia	.	1	3	2
CENTRAL NERVOUS SYSTEM				
Sub-arachnoidal haemorrhage	1	.	.	.
Suppurative meningitis/ meningo-encephalitis	3	1	.	.
MYOCARDIUM- Interstitial haemorrhage	.	.	1	.
LYMPHOID TISSUES				
Lymphoid depletion in lymph nodes	3	1	3	2
Reactive lymph nodes (oedema, inflammation)	2	1	4	6
Lymphoid depletion in spleen	1	.	2	1
Multifocal haemorrage in Peyer's patches	.	.	.	3
Reactive GALT	1	.	.	1
Congested spleen	.	1	.	1
LIVER				
Hepatic abscess	1	.	.	.
Multifocal suppurative hepatitis	1	.	1	1
INTESTINES - Bacterial enteritis	.	.	.	2
PERITONITIS	.	.	1	.
SKIN				
Cellulitis	.	12	.	5
Severe necrotizing dermatitis	2	.	.	.

Table 8.2 Histopathological lesions in New Zealand sea lion pups diagnosed with bacterial infection at post-mortem, during the epidemic (2001/2002 and 2002/2003) and post-epidemic (2003/2004 and 2004/2005) seasons. For each period, lesions are listed when bacterial infection was the primary cause of mortality ("primary lesions") and when it was only contributing to death ("secondary lesions").

congested vessels, with infiltration of large numbers of viable and degenerate neutrophils with variable numbers of plasma cells, lymphocytes and macrophages (Fig. 8.5a, 8.5b). Large numbers of Gram negative bacilli were present amongst these inflammatory cells and some of them occasionally contained intracellular bacilli, confirming ante-mortem bacterial infection. In more severely affected individuals (Fig. 8.5c), the meninges also contained abundant fibrin and haemorrhage, and inflammation extended into the underlying parenchyma. In most pups diagnosed with meningitis, microscopic examination of spleen and lymph nodes also showed general lymphoid depletion. *K. pneumoniae* was grown in pure culture from a swab of the spinal cord of one severely affected pup. This pup was also diagnosed with peritonitis and *K. pneumoniae* was similarly isolated from a swab of this lesion. Other histopathological findings associated with macroscopic lesions reported in the present study but not listed in Table 8.2, include acute to subacute suppurative arthritis, tenosynovitis and cellulitis of tissues surrounding the joint (Fig. 8.6a, 8.6b and 8.6c).

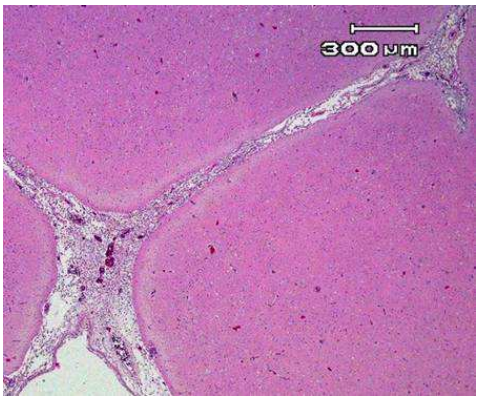

Figure 8.5a: Cerebral cortex of a NZSL pup. Meninges contain oedema fluid, inflammatory cells and bacteria. H&E.

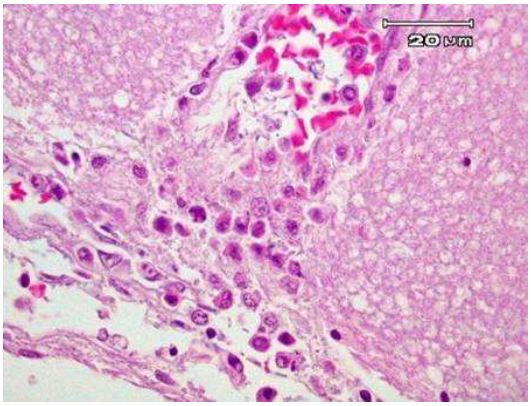

Figure 8.5b: Higher magnification of Fig. 8.5a showing bacteria, neutrophils, lymphocytes and plasma cells. H&E.

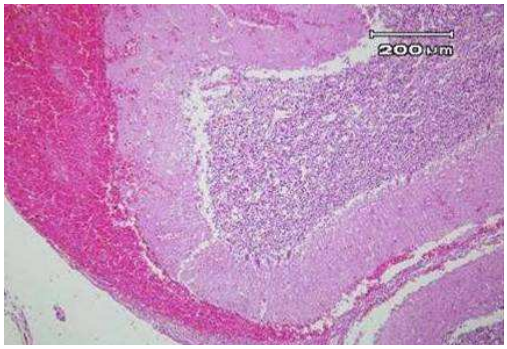

Figure 8.5c Cerebellum of a NZSL pup. Severe meningo-encephalitis and meningeal haemorrhage. H&E.

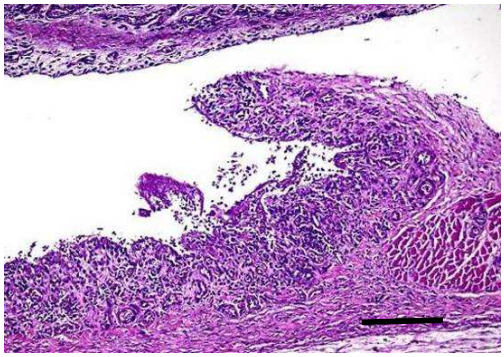

Figure 8.6a: Suppurative inflammation of the synovial membrane and underlying connective tissues of the carpal joint of a NZSL pup. H&E. Bar=200µm.

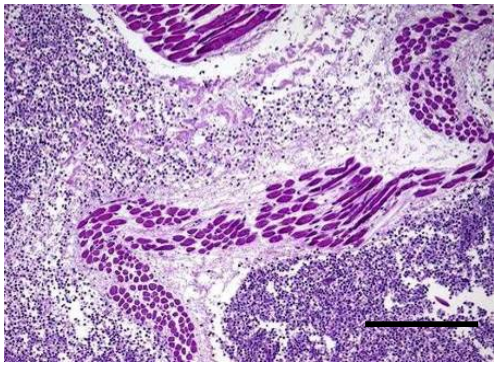

Figure 8.6b Higher magnification of Fig. 8.6a showing infiltration of inflammatory cells and bacterial colonies. H&E. Bar=40 µm.

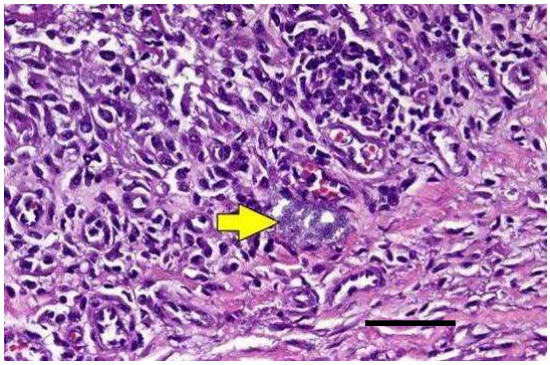

Figure 8.6c Extensive oedema, suppuration and necrosis surrounding and separating muscles bundles adjacent to an infected joint. H&E. Bar=150 µm.

The 2001/2002 and 2002/2003 NZSL breeding seasons at Sandy Bay Beach rookery were characterised by high neonatal mortality, with an abnormally elevated proportion of bacterial infections diagnosed at necropsy compared to previous years and those afterwards. *Klebsiella pneumoniae* was grown in pure culture from almost all of the pups found dead during the epidemics and to a lesser extent in the following years. Polyarthritis was the most frequent feature both during and after the epidemics but various other macro- and microscopic pathological changes were also observed.

Opportunistic bacteria such as *Klebsiella* spp. can take advantage of pre-existing conditions such as cutaneous wounds or debilitation due to viral or parasitic infections to cause severe lesions and septicaemia. The occurrence of such bacterial disease in pinniped pups could be explained by an underdeveloped immune system (Cavagnolo and Vedros, 1979; Kock, 2003). In addition, it has been reported that suckling pups would have a high predisposition to non-specific bacterial disease, secondary to starvation and reduced immunologic capability (Sweeney and Gilmartin, 1974; Cavagnolo and Vedros, 1979). There are numerous reports of opportunistic bacteria isolated from tissues of pinnipeds. For instance, *Klebsiella* spp. including *Klebsiella pneumoniae,* have been cultured from assorted tissues from stranded adult pinnipeds and from pups at necropsy, often in the absence of associated lesions (Sweeney and Gilmartin, 1974; Smith et al., 1974a; Vedros et al., 1982; Johnson et al., 1998; Thornton et al., 1998). Ubiquitous bacteria including the Gram negative *Klebsiella* spp. and *E. coli* and the gram positive *Streptococcus* spp. and *Staphylococcus* spp. are part of the environmental bacterial flora of the rookery and therefore are frequently cultured from cutaneous wounds and mucosal lesions (Sweeney and Gilmartin, 1974; Thornton et al., 1998) but are also found in the nasal cavity of clinically healthy animals (Hernández-Castro et al., 2005). In NZSL pups, the same opportunistic bacteria were isolated from cases of septic arthritis, cerebrospinal meningitis, septicaemia and cutaneous abscesses but *K. pneumoniae* predominated during the 2001/2002 and 2002/2003 epidemics. The isolation of *K. pneumoniae* from lesions of NZSL pups at necropsy was not surprising but the high prevalence and the number of deaths directly associated with this opportunistic pathogen suggested an unusual virulence.

During the epidemics at Sandy Bay rookery, *Klebsiella pneumoniae* was cultured from the majority of bacterial lesions. Before 2001/2002, bacterial arthritis in NZSL pups had been diagnosed only six times but there are no bacteriology data available for these cases. For the 2001/2002 and 2002/2003 breeding seasons, *K. pneumoniae* was isolated from all but one of the infected joints, which may reflect a high degree of environmental exposure by *K. pneumoniae* at that time. However, a variety of other bacteria were also identified in the 2004/2005 breeding season, including *Staphylococcus* spp. and *Streptococcus* spp. and the number of isolates of *K. pneumoniae* during that season showed a parallel decrease. It seems that other opportunistic bacteria had begun to supplant *K. pneumoniae* as the cause of bacterial arthritis in NZSL pups in 2003/2004 and 2004/2005. Presumably *K. pneumoniae* was still circulating in the rookery but pups could have developed some immunity against the pathogen, which could explain the decline of prevalence of lesions involving *K. pneumoniae*.

The pattern of bacterial lesions due to *Klebsiella pneumoniae* in NZSL pups is supportive of underlying neonatal septicaemia. Most pups had lesions of suppurative arthritis. Like other opportunistic enterobacteria, *K. pneumoniae* is known to be involved in septic arthritis in neonates of domestic animal species and of humans (Abuekteish et al., 1996; Ryckewaert and Bardin, 1996; Bernabé et al., 1998; Kao et al., 2003). However in NZSLs, arthritis could affect several distant joints in the same pup and was diagnosed with other concomittant bacterial lesions from which *K. pneumoniae* was also isolated. This provides some evidence for septicaemia being at the origin of poly-arthritis, rather than the aetiological hypothesis of trauma as suggested for young children with septic arthritis (Abuekteish et al., 1996; Ryckewaert and Bardin, 1996).

In most cases of neonatal septicaemia, the umbilicus is the route of entry of bacteria (Jubb et al., 1993) although respiratory and gastrointestinal tracts are also important routes. In some NZSL pups, it is possible that ingested *Klebsiella pneumoniae* were able to invade the intestinal mucosa at sites of pre-existing damage due to hookworm infestation. Systemic involvement may then have followed if the organisms were able to enter the circulation. However, *K. pneumoniae* has also been reported to cause haemorrhagic enteritis in rabbits (Coletti et al., 2001) and gastro-enteritis in chinchillas (Bartoszcze et al., 1990) in the absence of intestinal parasites. In NZSL pups, severe and

fatal skin lesions associated with *K. pneumoniae* occurred only during the epidemic seasons. Cellulitis was generally diagnosed with other internal bacterial lesions also caused by *K. pneumoniae*, suggesting that septicaemia could have induced these subcutaneous lesions. In contrast, necrotizing dermatitis was also diagnosed alone, indicating that the source of bacteria in this case was more likely to be environmental than haematogenous, with *K. pneumoniae* presumably entering after skin trauma. Cutaneous disease involving *K. pneumoniae* was reported only once before in an immunocompetent human (Park et al., 2004). In this case, acute cellulitis rapidly extended through both subcutaneous and cutaneous tissues but the authors suggested that the origin of bacterial infection in that case was likely to be environmental, despite the absence of cutaneous trauma. Similar brain lesions to those observed in NZSL pups were reported in monkeys with severe suppurative meningo-encephalitis due to *K. pneumoniae* (Postal et al., 1988). It is likely that infections with *K. pneumoniae* at least in wild animal populations, are a naturally occurring phenomenon (Giles et al., 1974; Jackson et al., 1980). In a naïve population, the introduction of a virulent pathogenic strain can result in fatal septicaemia or more localised but still fatal lesions.

CONCLUSION – *Chapter 8*

Bacterial epidemics at Sandy Bay Beach generated two consecutive seasons of high mortality in NZSL pups and *Klebsiella pneumoniae* was identified as the causal agent. The high prevalence of lesions induced by this pathogen and the high pup mortality during these two breeding seasons were a good indicator of the virulence of this bacteria, compared to other opportunistic pathogens causing lesions before the epidemics. *K. pneumoniae* was isolated from a range of suppurative lesions, but joints were the most commonly affected site. This pattern of joint lesions, in combination with involvement of tissues such as the meninges and peritoneal organs is typical of neonates with a bacterial septicaemia. After two years of epidemics, the prevalence of *K. pneumoniae* isolates has decreased but it seems that the pathogen is still circulating in the rookery and NZSL pups remain exposed to the pathogen, yet at a lower level than during the epidemics.

Chapter 9

Humoral immune response to *Klebsiella pneumoniae* in New Zealand sea lions, *Phocarctos hookeri* and investigation of the passive transfer of immunity to pups.

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to *Veterinary Microbiology*

ABSTRACT- *Chapter 9*

During the 2001/02 and 2002/03 breeding seasons, *Klebsiella* epidemics resulted in a dramatic increase of pup mortality in New Zealand sea lions (NZSL) on Enderby Island, Auckland Islands. To estimate the prevalence of infection in the NZSL population, a serological test was developed using a Western blot technique with a crude antigen derived from a *Klebsiella pneumoniae* isolate from a NZSL pup. All archived frozen serum samples collected at the Sandy Bay rookery (Enderby Island) from 1997/1998 to 2004/2005 were tested. Over this period, only 15.9% of NZSL pups between birth and five months of age (n=301) were sero-positive for anti-*Klebsiella* antibodies compared to 95.7% of adults (n= 290). There was no apparent change in prevalence as a result of the two epidemics in 2001/2002 and 2002/2003. In addition, a method for the determination of immunoglobulin G (IgG) levels in sea lion serum was developed to investigate passive immunoglobulin transfer to neonates and development of acquired immune response. The IgG concentration was low in pups (n=53) from birth to 5 months of age (median: 4.4 mg/ml) and did not show any significant increase with age over this time period. In adult females (n=44), IgG levels were significantly higher. Based on the serological results, it was not possible to determine whether *K. pneumoniae* was an endemic pathogen to the NZSL population because the test could not discriminate between *K. pneumoniae* and *K. oxytoca.* Given the low transfer of passive immunity observed in this study, pups in their first weeks of life seemed to be highly vulnerable to infectious agents, such as *K. pneumoniae*.

INTRODUCTION– *Chapter 9*

The New Zealand sea lions (NZSLs) are one of the world's rarest pinnipeds and breed almost exclusively on the Auckland Islands (50°S, 166°E). In addition, according to the latest estimates (between 10,000 and 12,700 individuals, Chilvers, unpublished data), their population size has been declining for the past few years. The 2001/2002 and 2002/2003 breeding seasons on Enderby Island (Auckland Islands) were marked by high pup mortality as determined by counts at the six week census date, 31.3% in 2001/2002 and 21.7% in 2002/2003 compared to the previous year (10.9%, Castinel et al., submitted *b*; *see* Chapter 2), with a dramatic increase of the proportion of bacterial infections diagnosed at necropsy (Wilkinson et al., 2006). *Klebsiella pneumoniae* was consistently isolated in pure culture from internal organs of dead pups (Castinel et al., submitted *c*; *see* Chapter 7). This bacterium belongs to Klebsiellae, a group of opportunistic enterobacteriacae that are frequently involved in nosocomial infections and associated with high morbidity and mortality in immuno-compromised humans (Gouby et al., 1994; Peña et al., 1998).

Bacterial infections are common in free-ranging pinnipeds, involving a wide range of opportunistic (Stroud and Roffe, 1979; Thornton et al., 1998) and potentially zoonotic micro-organisms, including *Klebsiella* spp. (Stroud and Roffe, 1979; Higgins et al., 2000; Hernández-Castro et al., 2005), but without necessarily causing death. On the other hand, epizootic events associated with the zoonotic *Leptospira* spp. have been reported in California sea lions (*Zalophus californianus*), periodically affecting the population dynamics (Gulland et al, 1996). *Klebsiella pneumoniae* had never been associated with mass mortality in any pinniped species before 2001/2002.

Bacterial culture is the standard method for phenotypic identification of *Klebsiella* infections in humans and animals. The detection of serum antibodies to *Klebsiella* spp. seems to be restricted to research applications rather than standard diagnostic serology, and most contemporary serological tests are based on the enzyme linked immunosorbent-assay (ELISA) (Sahly and Podschun, 1997). Even though enzymatic immunoassays have been widely used to investigate the exposure of pinnipeds to some enterobacteriacae such as

Salmonella spp. and *Brucella* spp. (Aschfalk et al., 2002; Nielsen et al., 2005), the Western blot (WB) technique can be used as an alternative to the ELISA. It has shown comparable sensitivity and specificity for serology against a variety of bacterial infections (Kittelberger et al., 1995a, 1995b). Unlike ELISA, it does not require the calculation of specific cut-off values which is an advantage in wild pinnipeds where experimental infections to validate the assay are not possible.

It is likely that *Klebsiella* antibody responses are frequently present in humans and animals even after infections have been cleared. The level of pup mortality on the Auckland Islands in 2001/2002 and 2002/2003 suggested that this may be a novel pathogen in NZSL recently introduced to a naïve population. Thus, we hypothesised that serum antibodies to *Klebsiella* may be absent or only present with low frequencies in the population before the 2001/2002 and 2002/2003 breeding seasons.

The aim of this study was to investigate the temporal variation and overall seroprevalence of anti-*K. pneumoniae* antibodies using a WB method, in the NZSL population on Enderby Island, before, during, and after two years of epidemics caused by this pathogen. Results from this study led to further investigations into the passive transfer of humoral immunity to sea lion pups, with preliminary results reported here.

Collection of samples

Blood samples for serology were collected from 240 pups and 16 older sea lions necropsied at Sandy Bay rookery (Enderby Island, Auckland Islands) during the 1997/1998 to 2002/2003 breeding seasons and in 2004/2005. Blood was taken from the heart to avoid any contamination when opening the carcass. It was then centrifuged at 7,000 rpm for 15 minutes and the serum was frozen in liquid nitrogen. Serum was also obtained from live sea lions: samples were collected from the caudal gluteal vein into sterile untreated glass tubes. Between 1997/1998 and 2004/2005, adult females were captured for ongoing studies (n=276 in Sandy Bay colony and n=10 on Dundas Island) and sampling of females was conducted under general anaesthesia using isofluorane/O_2 (Gales and Mattlin, 1997). In May 2000, 54 pups of approximately five months of age were manually restrained and blood was collected as above. In 2004/2005, 20 serum samples were obtained from pups of different ages, varying from birth until the end of the period of study when the majority of pups were two months-old; some individuals were sampled twice or three times resulting in a total of 32 samples. The age of live pups was known with accuracy since they were identified and marked at birth with a plastic cap; however, the age of dead pups was estimated by the difference between the annual mean birth date (26th of December, Chilvers et al., 2006a) and the date of necropsy (Table 9.1). For analysis, pups other than five months-old were divided into two groups (from birth to one month-old and from one to three months of age).

Bacterial strains

Each *Klebsiella* isolate had been cultured from the internal organs of different pups. Tissue samples were initially incubated on Sheep Blood agar plates at 37˚C for 24 to 48 hours to allow sufficient growth. Phenotyping tests, consisting of Gram stain and standard enzymatic tests (Microbact^{TM} MedVet Science Pty Ltd, Adelaide, Australia), were conducted on pure cultures to identify the isolates. These were typed as: *Klebsiella pneumoniae* 1; *Klebsiella pneumoniae* 2 and *Klebsiella oxytoca*. Pulsed-field gel electrophoresis (PFGE) previously conducted on *Klebsiella* isolates from NZSL showed

that *K. pneumoniae* 1 and 2 isolated during the epidemics from different pups were clonal (*see* Chapter 7, Fig. 7.2) whereas *Klebsiella oxytoca* that was isolated in 2000/2001 before the epidemics was unrelated to these two *K. pneumoniae* isolates (Castinel et al., submitted *c*; *see* Chapter 7).

		1997/ 1998	1998/ 1999	1999/ 2000	2000/ 2001	2001/ 2002	2002/ 2003	2004/ 2005	*total*
live pups	Birth to 1 month-old	-	-	-	-	-	-	23	23
	1 to 3 months-old	-	-	-	-	-	-	9	9
	approx. 5 months-old	-	-	55	-	-	-	-	54
dead pups	Birth to 1 month-old	-	23	27	33	54	8	9	154
	1 to 3 months-old	23	-	7	11	19	23	3	86
adult females	Live (captures)	43	40	53	17	22	66	45	286
	Necropsy	2	3	1	4	3	2	1	16

Table 9.1: Distribution of serum samples from New Zealand sea lions by age, breeding season and survival, between 1997/1998 and 2004/2005 on the Auckland Islands. There were no data available for the 2003/2004 breeding season.

Preparation of antigens

Antigens were prepared from the three *Klebsiella* isolates by mixing 0.5g of washed bacterial pellet with 4.5ml of polyacrylamide gel electrophoresis (PAGE) sample buffer (62mM Tris HCL [pH 6.9], 5% sodium dodecyl sulphate (SDS), 1% mercaptoethanol (ME), 10% glycerol), by heating at 95 ºC for 10 minutes and finally by centrifuging at 10,000 rpm for 15 minutes. The supernatants were used as antigens. The crude antigens derived from the three isolates were named AG1 (*K. pneumoniae* isolate 1), AG2 (*K. pneumoniae* isolate 2), and AG3 (*K. oxytoca* isolate). Aliquots of the crude antigens were incubated with proteinase K (pK, at 60mAU/ml) at 56ºC for one hour and then analysed by SDS-PAGE using Coomassie and silver staining methods (Kittelberger et al., 1994).

SDS-PAGE and Western blot

SDS-PAGE was performed on 4-15% polyacrylamide gradient gels (Ready-gels, Tris-HCl, Bio-Rad, Hercules, CA) with a discontinuous Tris-glycine buffer system in a Mini-

Protean II electrophoresis cell (Bio-Rad, Hercules, CA). Gels were run for 40 minutes at 200V and then either used for Western blotting or for gel staining (Kittelberger et al., 1994). For the analysis of antigens, gels with 10 or 15 wells were used, while preparative gels were preferred for the screening of serum samples for anti *Klebsiella* antibodies (Klebs-WB).

Electrophoretic transfer of separated antigens from polyacrylamide gels onto membranes (Immobilon P membrane, Millipore, Bedford, MA) was carried out in a Trans-Blot SD cell (BioRad, Hercules, CA) at 15V for 20 minutes. Membranes were blocked overnight at room temperature (RT) in TBS (50mM Tris-HCl, 150mM NaCl, 0.01% merthiolate, pH 7.5) containing 3% non-fat dry milk powder (Anchor, New Zealand; TBS-MP). NZSL serum samples, diluted 1:200 in TBS-MP were incubated at RT for two hours on the membranes in a miniblot system (Immunetics, Cambridge, MA). The membranes were washed three times in TBS containing 0.05% Tween-20 (TBS-T), followed by incubation at RT for 30 minutes with protein A/G-alkaline phosphatase conjugate (Immunopure Protein A/G, Pierce Rockford, IL) diluted 1:5,000 in TBS-MP. After three washes with TBS-T, the membranes were incubated with the substrate NBT-BCIP (Roche Diagnostics, Mannheim, Germany) for three minutes at RT, followed by two washes with deionized water.

Molecular weight (MW) markers (high-range rainbow molecular weight markers Amersham-GE Healthcare, Bucks, UK) were run on every gel. In the Klebs-WBs, a protein A-purified rabbit anti-*K. pneumoniae* antibody (B65891R, 4-5 mg/ml, Biodesign, Saco, ME) was run as positive control at dilutions of 1:5,000, 1:20,000 and 1:80,000 in TBS-MP. Assuming that the majority of IgGs was anti-*Klebsiella*, this corresponds to 0.8-1 μg/ml, 0.2-0.25 μg/ml and 0.05-0.06 μg/ml respectively. As a negative control, serum from a dead NZSL pup was run at 1:200 dilution in TPS-MP. The pup had died from traumatic injury prior to the epidemic years. None of the internal organs (lungs, liver, spleen, several lymph nodes, thymus and faeces) were positive on bacterial culture. This serum did not show any staining in the Klebs-WB. Intensity of staining in the Klebs-WB was coded as follows:

negative =0; positive + (positive control 1:80,000) =1; positive ++ (positive control 1:20,000) =2 and positive +++ (positive control 1:5,000) =3.

Serum immunoglobulin G (IgG) concentrations

For the determination of serum IgG concentrations, a WB method was developed (IgG-WB) based on a similar method for bovine IgGs (Kittelberger, unpublished data). Sea lion IgG standards were prepared from NZSL adult female serum samples by ammonium sulphate (AS) precipitation. The IgG concentration was determined using the QuickStart protein assay (BioRad, Hercules, CA). The purity of the IgG preparation was checked by SDS-PAGE and Coomassie staining.

For the IgG-WB, serum samples were diluted in SDS-PAGE sample buffer at 1:10 for pups and 1:40 for older sea lions. Serum samples and IgG standards were run at concentrations of 1.6, 0.8, 0.4, 0.2 and 0.1mg/ml (diluted in SDS-PAGE sample buffer) on every gel. SDS-PAGE was performed on 15 well 4-15% polyacrylamide gradient gels under the same conditions as already described (see 2.4). Electrophoretic transfer was performed as for the Kleb-WB. After blocking with TBS-MP, membranes were incubated with protein A/G-alkaline phosphatase conjugate, followed by substrate incubation under the same conditions as for the Klebs-WB. IgG concentrations were calculated from blot images using the program Quantity One (Bio-Rad, Hercules, CA).

Data analyses

For statistical analysis, five age classes were defined for the NZSLs: less than one month-old (1), between one and two months-old (2), between two and three months-old (3), five months-old pups (4) and older animals including yearlings, sub-adults and adults grouped together (5).

The frequency of the four possible responses testing the Klebs-WB (0, 1, 2 and 3) was analysed by year and by survival class (0: dead and 1: alive) with the FREQ procedure in SAS (Statistical Analyses System® 9.1.3, SAS Institute Inc., Cary, NC, USA) separately for pups and for adults. The effect of year and survival on the response variable

"serological test" was investigated with a generalized logits model (LOGISTIC procedure with GLOGIT option in SAS).

IgG concentrations were reported graphically only with an estimation of the median for the pups. When concentrations were too high to be calculated with the standard curve, the individual was given the maximum concentration allowed by the method (40mg/ml for pups and 80mg/ml for adults). The same rule applied to concentration beyond the lowest limit (1 mg/ml for pups and 2 mg/ml for older sea lions). Data are presented as means. A statistical difference was assumed when $P<0.05$.

Optimisation of the WB methods

At first, a suitable anti-sea lion Ig conjugate had to be identified. Using the IgG-WB method, it was found that a fusion protein A/G-alkaline phophatase conjugate reacted strongly with sea lion IgGs and IgGs of many other pinniped species (data not shown). For the detection of anti-*Klebsiella* serum antibodies by the Klebs-WB, three antigens were initially compared, which had been prepared from two *K. pneumoniae* isolates and one *K oxytoca* isolate from Auckland Islands' sea lions (AG1, AG2 and AG3). Antigens were analysed by SDS-PAGE using a combination of undigested and pK-digested antigens with Coomassie staining (proteins) and silver staining (polysaccharides) (Figure 9.1). In the Coomassie-stained gels, the undigested antigens showed numerous distinct protein bands over a wide molecular weight range (20-100kDa). Protein bands disappeared completely in the Coomassie-stained gel after digestion. Digested antigen preparations still showed strong staining in the silver-stained gels. The diffuse staining over a wide MW range (about 20 – 200 kDa) is typical for smooth lipopolysaccharides (SLPS) of *Klebsiella* spp. (Tomás et al., 1986) and other enterobacteriacae (Kittelberger et al., 1995b). All three bacterial strains showed very similar staining patterns for all combinations of treatments.

After optimisation of antigen concentrations and conjugate dilutions and by using a panel of 28 different NZSL serum samples, it was found that staining patterns for the three *Klebsiella* antigen preparations were identical, i.e. the density of staining in sera with high, medium and low titres was the same for all three antigens. Furthermore, undigested and digested antigens showed strong diffuse staining over a wide MW range (about 20-200kDa), typical for SLPS. Non-digested antigens showed extra distinct bands of low MW, presumably protein bands (data not shown). If staining of protein bands had been present in the same MW range as the SLPS staining, it would have been masked. Therefore, for the analysis of large numbers of NZSL serum samples, an optimized Klebs-WB was used, using pK-digested *K. pneumoniae* antigen 1. A typical test run image is shown in Figure 9.2. Conditions for the detection of IgGs by the IgG-WB were also optimized by testing various concentrations of sea lion serum and conjugate (data not shown).

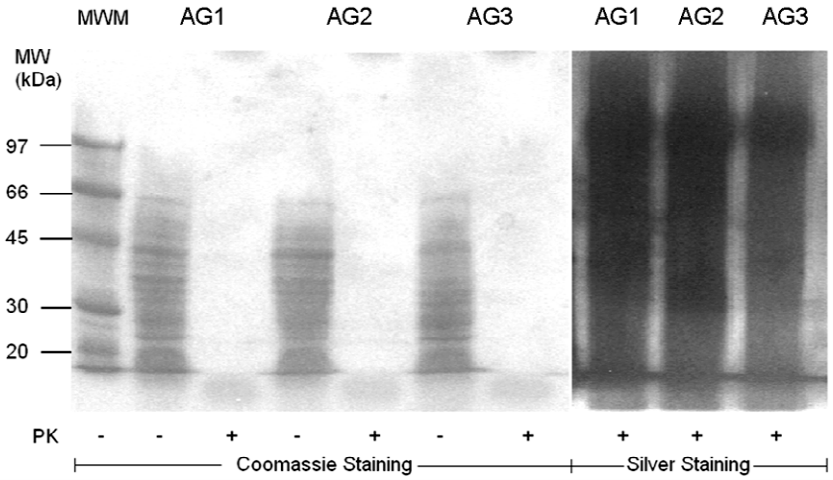

Figure 9.1 Analysis of various *Klebsiella* antigen preparations on polyacrylamide gels. Molecular weight markers (MWM) are on the left. Antigens 1 and 2 (AG1 and AG2) were prepared from isolates of *Klebsiella pneumoniae* and Antigen 3 (AG3) from *Klebsiella oxytoca*. Antigens incubated with proteinase K (PK) are noted "PK+" and the others "PK-".

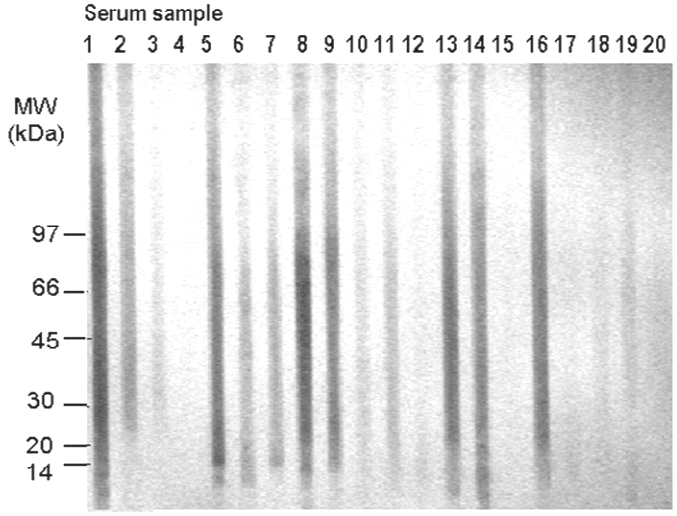

Figure 9.2 Example of an optimized Western Blot for the detection of antibodies to *Klebsiella pneumoniae* in New Zealand sea lions (NZSLs). Each lane represents a single serum sample. Lanes 1 to 3 are dilutions of a rabbit-anti *Klebsiella* control serum. Lane 4 is the negative control. Lanes 5 to 20 are NZSL lion sera. As an example, lane 5 is positive-grade 3(+++), lane 6 is positive-grade 2 (++), lane 10 is positive-grade 1(+) and lane 17 is negative.

Klebsiella serology results

An example of a typical Klebs-WB for 16 NZSL sera is illustrated in Fig. 9.2, underlining the contrast between positive responses of different intensity. The staining of positive serum samples and the positive control is typical for SLPS. The strength of staining in the Klebs-WB was graded according to the positive control antibody used.

Frequency of sea lions that tested positive for circulating anti-*Klebsiella* antibodies is shown in Fig. 9.3 and for pups in Table 9.2. Neither year nor survival had a significant effect on the response to the serological test. There was a higher prevalence of pups presenting anti-*Klebsiella* antibodies in 2004/2005 compared to the previous years except for 1998/1999 but this was not significant. The age of pups significantly influenced the presence of antibodies ($P<0.0001$). In Table 9.3, the intensity of the anti-*Klebsiella* serological response is presented for live pups when the age was precisely known. No statistical analyses were undertaken as numbers per category were too small. However, there was no Klebs-WB strength 3 response in this subpopulation, regardless of the age (Fig. 9.3).

The prevalence of anti-*Klebsiella* antibodies in 16 dead adults (Fig. 9.4) did not significantly vary between years. However, because of the small sample size compared to the overall population of adults tested (n=306), year ($P<0.0001$), but not survival, appeared to influence significantly the intensity of the serological response in the model. Prevalence of the presence of anti-*Klebsiella* antibodies in adults oscillated between 85.7% in 1997/1998 and 100% in 2000/2001 and 2001/2002 (Fig. 9.4).

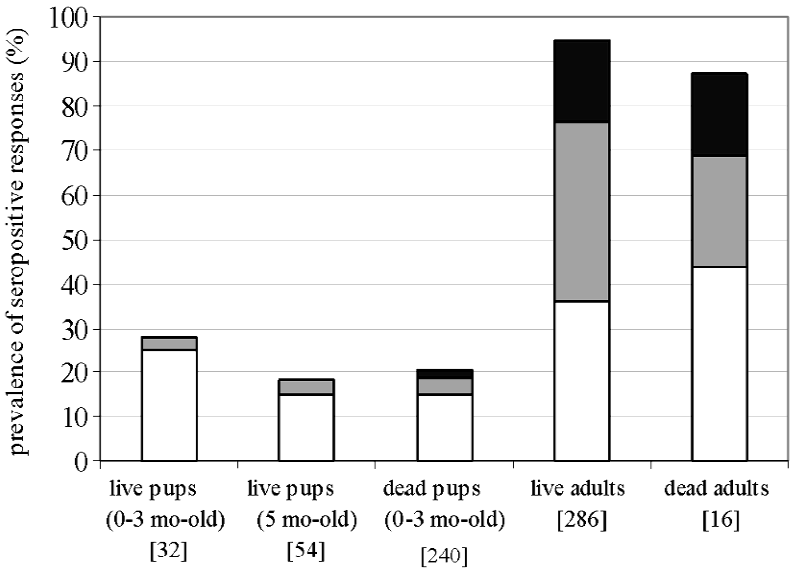

Figure 9.3 Distribution of the intensity of positive responses of adult New Zealand sea lions (NZSLs) and pups tested for anti-*Klebsiella* serum antibodies, from 1997/1998 to 2004/2005.The proportion of intensity of the reaction between NZSL sera and the test response is presented as ascending grades: grade 1 (white), grade 2 (grey) and grade 3 (black). The number of NZSL by group is indicated between brackets.

Year	1998/1999	1999/2000	2000/2001	2001/2002	2002/2003	2004/2005
Prevalence of seropositive pups	39.1% (9/23)	18.2% (16/88)	11.4% (5/44)	6.8% (5/73)	3.3% (1/30)	27.9% (12/43)

Table 9.2 Prevalence of New Zealand sea lion (NZSL) pups (live and dead) with anti-*Klebsiella* serum antibodies from 1998/1999 to 2004/2005.

Intensity of test response in pups	1 week-old (n=10)	2 weeks-old (n=6)	3 weeks-old (n=3)	4 weeks-old (n=4)	7 weeks-old (n=4)	8 weeks-old (n=5)	9 weeks-old (n=1)	5 months-old (n=54)
0	100%	100%	100%	100%	25%	0%	0%	81%
1	0%	0%	0%	0%	75%	80%	0%	15%
2	0%	0%	0%	0%	0%	20%	100%	4%
3	0%	0%	0%	0%	0%	0%	0%	0%

Table 9.3 Prevalence of anti-*Klebsiella* serum antibodies in live New Zealand sea lion (NZSL) pups. Individuals from 1 to 9 weeks-old were sampled during the 2004/2005 breeding season whereas samples from pups of approximately five months of age were collected in May 2000. Absence of antibodies was scored as 0, the ascending intensity of positive responses is scored from 1 to 3.

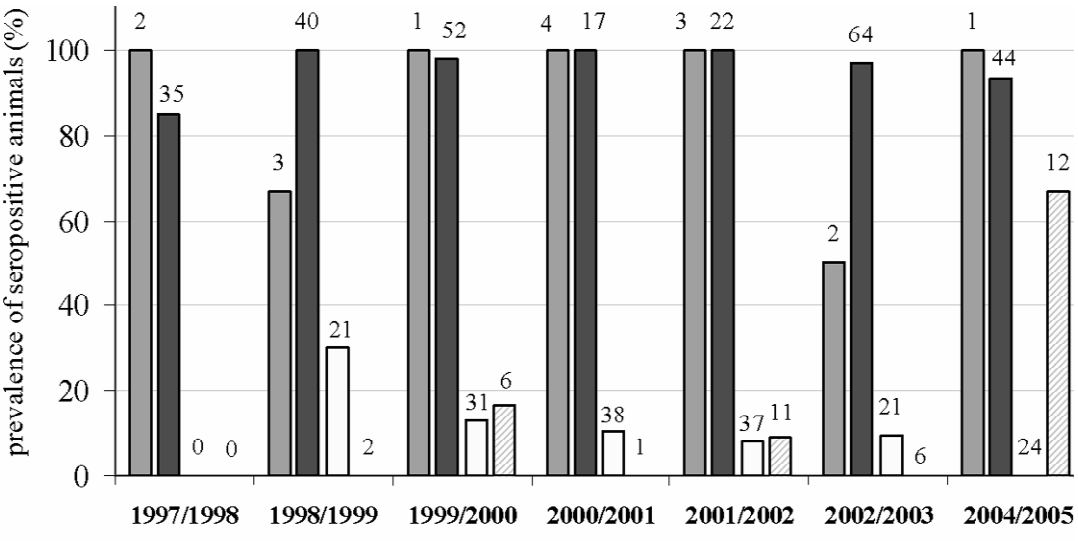

Figure 9.4 Detection of anti-*Klebsiella* serum antibodies in adult New Zealand sea lions and pups, from 1997/1998 to 2004/2005. The number of animals per category is indicated above the chart bars. Categories are as follows: live adults (black), dead adults (grey), pups from birth to one month-old (white) and pups from 1 to 3 months-old (grey stripes). Data from dead and live pups were pooled for this graph.

IgG serum concentrations

A typical example of an IgG-WB run is shown in Fig. 9.5. The IgG band had an approximate MW of 130kDa. As can be seen from the IgG standards, the absolute concentrations detectable by this method ranged from 0.1mg to 1.6mg/ml. The lower limit in the test runs performed was 1 mg/ml and the upper limit was 80 mg/ml. The limitation would be the amount of total protein, especially serum albumin, in the sample, which could lead to deterioration of bands during the electrophoresis run. There is theoretically no limit towards higher serum IgG concentrations by simply diluting serum samples further.

Serum IgG concentration results from a limited number of animals are presented in Fig. 9.6 for pups and in Fig .9.7 for adult sea lions. IgG concentration ranged between less than 1 mg/ml and more than 8 mg/ml in pups (median: 4.4 mg/ml) and between 2 mg/ml and more than 80 mg/ml in adults. Because only a limited number of serum samples were tested, no statistical comparison could be undertaken. However, the graphs show some evidence for a true difference existing between the two groups with adult concentrations being higher than for the pups (Fig. 9.6).

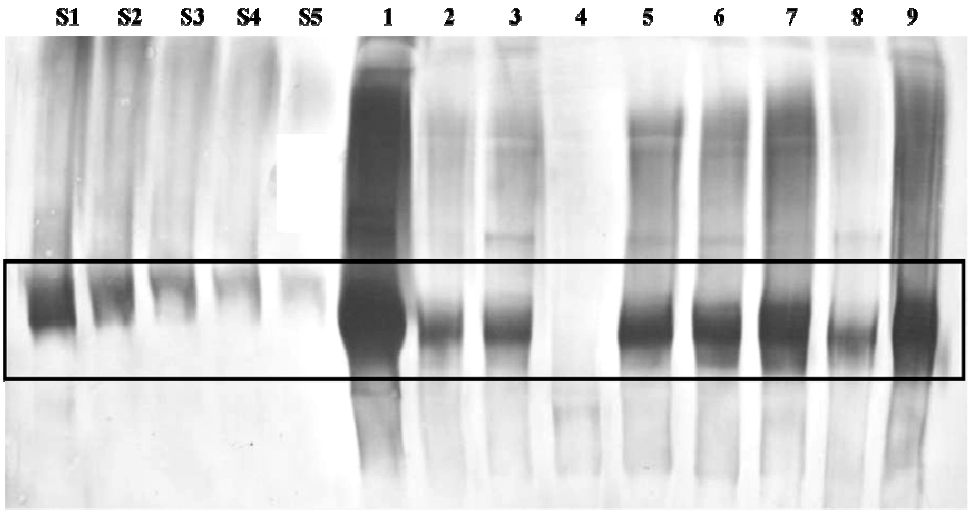

Figure 9.5 Example of a Western Blot for the detection of total immunoglobulins (Ig) G in New Zealand sea lion (NZSL) sera. Lanes S1 to S5 are purified IgG for a NZSL adult female at decreasing concentrations of 1.6, 0.8, 0.4, 0.2 and 0.1 mg/ml respectively. Lanes 1-9 are serum from different NZSLs. As for examples, IgG concentration in lane 1 is superior to 1.6 mg/ml by the dilution factor of the serum tested. In contrast, IgG concentration in lane 4 is inferior to 0.1 mg/ml by the dilution factor of the serum tested in this lane.

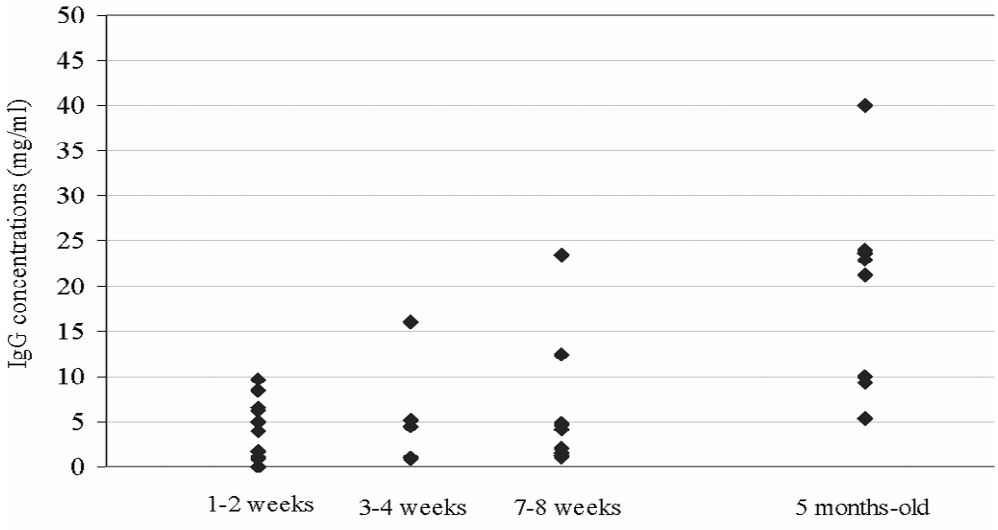

Figure 9.6 Total immunoglobulin (Ig) G concentration in New Zealand sea lion pups captured at Sandy Bay colony in May 2000 for animals of approximately five months of age pups and during the 2004/2005 breeding season for pups between 1 and 8 weeks-old. Each data point represents a pup.

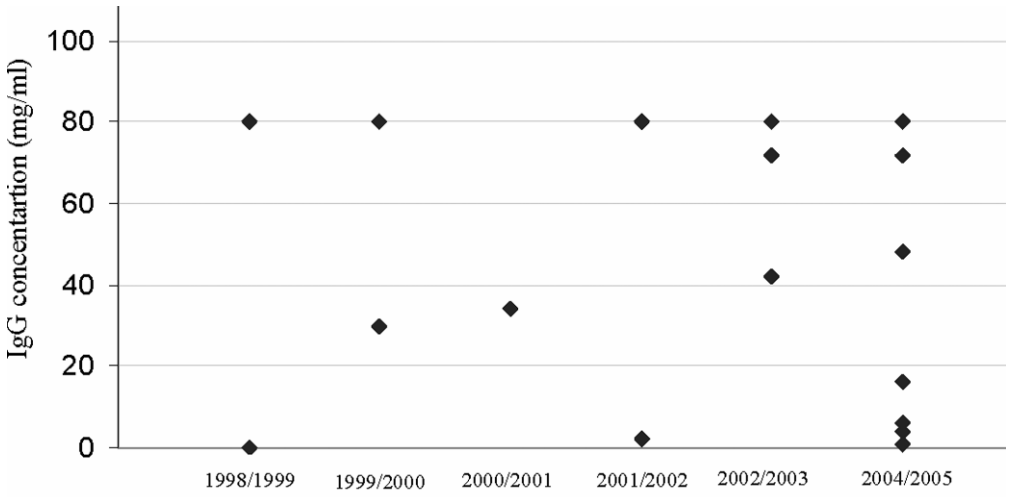

Figure 9.7: Total immunoglobulin (Ig) G concentration in New Zealand sea lion adult females captured at Sandy Bay rookery over the period 1999/2000 to 2004/2005. Each data point represents a female.

This study endeavoured to identify differences in the serological prevalence of anti-*Klebsiella pneumoniae* antibodies in the Auckland Islands' NZSL population before, during and after a *K. pneumoniae* epidemic, using more than six hundred archived serum samples that had been collected over eight years. Even if *Klebsiella* spp. are common environmental Gram-negative bacteria worldwide, it was hoped that differences in the seroprevalence over time could be identified because of the geographical isolation and possible lack of exposure of the NZSL population, and also of the magnitude of the epidemics that occurred in 2001/2002 and 2002/2003.

The serological test method chosen for the detection of anti *Klebsiella* antibodies was a Western immunoblot, rather than an ELISA. The major reason for this was that with the WB, immuno-dominant antigens could be identified when using a crude antigen preparation. For deciding on a cut-off in an ELISA, a panel of negative NZSL serum samples would have been needed, which were not available at the time. The Klebs-WB not only identified sero-positive and sero-negative sea lions but also showed that the majority of serum antibodies were directed against SLPS or the O-chain of SLPS.

During the developmental stage of the Klebs-WB it became clear that there was no difference in the response against the crude antigen preparations from *K. pneumoniae* and *K. oxytoca* when using the NZSL serum samples and the rabbit-anti *K. pneumoniae* commercial positive control antibody. Cross–reactivity between *Klebsiella* spp. has long been recognised (Sechter et al., 2000). Rissing et al. (1978) reported that human patients infected with a certain *Klebsiella* serotype showed antibodies against another serotype in the ELISA test. To overcome such limitation with cross-reactions, Brisse et al. (2004) proposed PCR-amplification of genes coding for capsular antigens as an alternative. Even though the use of PCR appears more accurate by combining high specificity and high sensitivity, it is not a suitable method for screening large numbers of archived serum samples. In this, the Klebs-WB used here could only be seen as a simple serological method for the detection of anti-*Klebsiella* antibodies but it turned out to be very sensitive

based on the amount of rabbit-anti *K. pneumoniae* antibody detectable, which was as low as 0.8µg/ml.

With the Klebs-WB, anti-*Klebsiella* antibodies were detected in all age classes of NZSL before, during and after the *K. pneumoniae* epidemics. Such antibodies were present even if the pathogen had not been identified in bacterial culture from tissues of necropsied sea lions prior to the epidemics (464 pups and 16 older animals), nor from adult sea lions caught and killed during the annual squid fishery season around the Auckland Islands (Duignan, unpublished data). Although the prevalence of pups testing positive for anti-*Klebsiella* antibodies was significantly lower than for adults, it still underlined the presence of such antibodies in pups before the first season of *K.pneumoniae* epidemics in 2001/2002. However, as indicated above, the test could not distinguish between the isolates of *K. pneumoniae* implicated in the epidemics and *K. oxytoca*. Thus, we cannot use this test to determine whether a novel pathogen was introduced into the population in 2001/2002 or not.

The seroprevalence of anti-*Klebsiella* antibodies in pups was generally low in all years except 2004/2005, but there was no significant difference between years or between dead and live pups. However, a significant difference by age was revealed by the higher seroprevalence in the oldest pups, especially in 2004/2005. In this season, data from necropsied pups contributed for only one third of the number of samples. It is very likely that the higher proportion of seropositive pups observed that year (Fig. 9.4) may be due to the inclusion of a majority of live pups in relatively good health compared to the previous years, where only serum samples from dead pups were analysed. A possible explanation is that pups that died acutely of *Klebsiella* septicaemia during the epidemics did not have sufficient time to seroconvert before they died.

In live pups monitored during the 2004/2005 season, the first seropositive result was recorded during the seventh week. By the eighth week, all pups showed anti-*Klebsiella* antibodies. In contrast, amongst the pups sampled in May 2000, when they were approximately five months-old, only 19% were sero-positive. This is very low when

compared to the seroprevalence in live adults for the same year (almost 100%) and still lower than for live pups in 2004/2005. This implies that neonates in 2004/2005 had been under higher exposure to *Klebsiella* antigens than older pups before the epidemics.

A possible reason for the sero-positivity of NZSLs before the epidemics could be the serological response to other *Klebsiella* spp. such as *K. oxytoca* which was isolated from one pup found dead at Sandy Bay Beach rookery in 2000/2001. The identity of this isolate was confirmed by using phenotyping tests and pulsed-field gel electrophoresis (PFGE) (Castinel et al., submitted *c*; *see* Chapter 7). Even though this isolate differed from that involved in the mass mortality of the following year, it suggests that this genus was at least present in the environment or circulating within the breeding population before the epidemics. Further isolates of *K. oxytoca* have been obtained from dead adult sea lions caught by the fisheries around the Auckland Islands since 1997 (Duignan, unpublished data). Only one PFGE type of *K. pneumoniae* was present during the epidemics further supporting the assumption that the pathogen was a novel introduction in 2001/2002 (Castinel et al., submitted *c*; *see* Chapter 7).

The lower frequencies of *Klebsiella* antibodies in NZSL newborns than in adults suggested that passive transfer of maternal anti-*Klebsiella* antibodies may be deficient. It also suggests that pups are born with a relatively immature immune response and take some time to respond to a bacterial challenge. Consequently, a WB method was developed for the quantification of sea lion serum IgGs and was used on a limited number of serum samples. While numerous sandwich ELISAs are commercially available for the determination of immunoglobulin levels in a number of animal species, no such ELISA existed for NZSL immunoglobulins. The IgG-WB method was able to detect serum IgG levels as low as 1 mg/ml.

In one to two weeks-old pups, IgG levels were found to be below 10 mg/ml whereas adults had much higher concentrations, generally more than 20 mg/ml. Such low levels of immunoglobulins in newborns could reflect a poor or failing passive transfer of immunity from the adult females. Pups obtain antibodies from their dam either by transplacental

migration or via colostrum and uptake of immunoglobulins from the small intestine. Pinnipeds and terrestrial carnivores share the same endotheliochorial placentation (Carter and Enders, 2004). Due to the limited number of studies investigating the role of transplacental transfer of antibodies in pinnipeds, it can only be suggested that prenatal transfer of antibodies may present some similarities between species. The transferred IgGs would then directly reflect the pup serum level measured at birth. For instance, the amount of maternal IgGs passively transferred via the placenta was estimated to be less than 5 % of the concentration of immunoglobulins of lactating females of the northern fur seal (*Callorhinus ursinus*) (Cavagnolo and Vedros, 1979) and of the harbour seal (*Phoca vitulina*) (Ross et al., 1994). Colostrum was reported to contain high levels of IgG in both phocids and otariids, and therefore to be the major source of IgG for harbour seal pups (Ross et al., 1994), grey seal pups (*Halichoerus grypus*) (Carter et al., 1990), northern fur seal pups (Cavagnolo and Vedros, 1979) and northern (*Mirounga angustirostris*) (King et al., 1998) and southern elephant seal pups (*M. leonina*) (Marquez et al., 2003). Although the serum levels of IgG in neonatal bovine calves was reported to be directly proportional to the colostrum concentration (Stott et al., 1979), high immunoglobulin concentrations of pinniped colostrum were not associated with high serum levels of IgG in northern fur seal pup (Cavagnolo and Vedros, 1979). Colostrum has not been analysed in NZSLs but the presence of IgG in the neonate serum indicated that passive transfer of immunoglobulins had occurred, yet at a low rate. The number of pregnancies in adult females could also condition the transfer of immunity: for instance the colostrum of primiparous northern fur seal females appeared to contain less Igs than in older dams (Beckmen et al., 2003). However, this did not apply to the present study on NZSLs, since all dams of the pups examined for IgG concentrations were known to be multiparous.

Overall, serum IgG levels in NZSL pups were consistent with those reported in other pinniped species. IgG concentrations in grey seals increased from 4.11 mg/ml during the first days of life to 10.66 mg/ml at five weeks-old (Carter et al., 1990). Higher values were reported in northern fur seals with 52.3 mg/ml at 3 days-old and 46.5 mg/ml (Cavagnolo and Vedros, 1979). In NZSLs, IgG concentrations ranged between less than 1.0 and 9.7 mg/ml from birth to two weeks-old, and it measured up to 16.1 mg/ml at four weeks of age

and up to 23.5 mg/ml at seven weeks. Values of NZSL pups between four and seven weeks-old overlapped with the standard reference range for canine pups (10-20 mg/ml, Foale et al., 2003). However, the average IgG concentration in NZSL pups from birth to about two months-old (4.4 mg/ml) remained below the normal values for canine pups. When NZSL pup IgG concentrations were compared to adult pinnipeds, higher levels were observed in adult grey seals at 70.6 mg/ml (Carter et al., 1990), in harbour seals at 20.0 to 90.0 mg/ml (Carter et al., 1992), in northern fur seals at 180.6 to 193.6 mg/ml (Cavagnolo and Vedros, 1979) and in adult NZSLs (80 mg/ml, present study). This suggests there is an increase of immunoglobulin production with age but there were no data available from NZSL yearlings and juveniles to compare with values for pups and adults in the present study.

Pups benefit from maternal immunoglobulins for a limited period of time whilst they develop their own immune response to the pathogens they are exposed to. There are no techniques available to discriminate maternally-derived from innate Igs but it is possible to determine the level of serum antibodies in the young animals after experimental challenge of their immune system induced by vaccination. Studies of response to vaccination in various pinniped species established that neonates are capable of developing a rapid, strong and highly specific humoral immune response to pathogens such as agents of equine tetanus (Beckmen et al., 2003) and rabies (Ross et al., 1994). Although NZSL pups showed no antibodies to *K. pneumoniae* for the first four weeks, some IgGs were detected by seven weeks suggesting that they had developed their own humoral immune response over that time, which appears longer than what was reported for northern fur seal, harbour seal and northern elephant seal neonates after immunological challenge (Beckmen et al., 2003; Ross et al., 1994; King et al., 1998).

Failure to develop protective immunity to a new antigen was illustrated in harbour seals during an outbreak of phocine herpesvirus-1 (King et al., 2001). In this study, existing antibodies directed towards a similar, but slightly different antigenic epitope bound to the pathogen and provided some protection but there was no evidence of a humoral immune response to the new virus in pups that died from the viral infection. Since NZSL neonates

rely upon maternal Igs and given that these are passed in small quantity through the colostrum, it is very likely that they were not protected against *K. pneumoniae* in 2001/2002 and 2002/2003. Most neonates died acutely, probably soon after they were exposed to an overwhelming challenge of the bacteria at Sandy Bay Beach rookery. There are still many unknown factors that may have contributed to the *K. pneumoniae* epidemics. Nevertheless, unlike the relative cross-protection previously evoked in harbour seal pups, serological results from the present study demonstrated that cross-reacting antibodies between *Klebsiella* species, already circulating in the adult NZSL population, were not transferred in large quantities and there was consequently limited protection for the pups during the epidemics.

CONCLUSION – *Chapter 9*

Because of the antigenic similarities between Klebsiellae species, it was not possible to conclude from the serological results whether *Klebsiella pneumoniae* was in fact endemic or if it had suddenly appeared on the rookery in 2001/2002. Although there was some evidence of a humoral immune response directed against *K. oxytoca* prior to the epidemics, it did not seem sufficient to protect the population against a pathogenic strain of *K. pneumoniae*. In addition, the present study suggested that seroconversion in response to *Klebsiella* antigens in the NZSL population had not been enhanced by the 2001/2002 and 2002/2003 epidemics. While many pups did not have any anti- *Klebsiella* antibodies, it seemed that those with such antibodies were not protected against *K. pneumoniae* infection. This implies that the NZSL population is still at risk from this pathogen, even after two epidemic seasons. Such an unfortunate paradox was also observed in harbour seals following epizootics with Phocine Distemper Virus (Carter et al., 1992).

Chapter 10

General Discussion

The research presented here on the causes of neonatal mortality in NZSLs has highlighted two interesting aspects. First, it showed that the main causes of mortality at Sandy Bay Beach on Enderby Island were being diagnosed in the same chronological order, for every breeding season. Secondly, it underlined the relative vulnerability of this NZSL population to unusual pathogens such as *Klebsiella pneumoniae.* Uncinariosis was considered to be a primary cause of death in about 13% of pups but all were found to be infected with hookworms. Consequently, determining the role of these parasites in neonatal mortality was complex but a comparative study involving artificially parasite-free and naturally infected pups allowed the investigation of the effect of hookworms on pup health.

I. Hookworms and pinniped neonates

Uncinaria spp. was the only endoparasite found in NZSL pups over the period of study, which is consistent with what has been observed in other pinnipeds (Lucas, 1899; Baylis, 1947; Olsen, 1952; Dailey and Hill, 1970; Botto and Mañé-Garzón, 1975; Berón-Vera et al., 2004). The hookworm species described in NZSLs presented some morphometric differences from the two other species already reported in pinnipeds (Baylis, 1933, 1947) and it is suggested to be at least a separate strain if not a different species. This raises the possibility that other epidemiological aspects might also be different. However, molecular characterisation is required to fully determine if the hookworms infecting NZSL pups are a novel species.

Studies undertaken in the current research on the life cycle of hookworms in NZSLs have shown similarities with *Uncinaria lucasi* in northern fur seals and California sea lions (Lyons, 1963; Lyons et al., 2000a). Although based on observations in three NZSL newborns, it is evident that these pups were ingesting L_3s through the colostrum. Further evidence supporting transmammary transmission was the finding of ten larvae of similar size and morphology in the mammary glands of lactating adult females.

Faecal examinations demonstrated that pups were shedding large numbers of hookworm eggs on the beach from three weeks of age indicating the minimum prepatent period would be about this long. It is hypothesised that these eggs will then develop into free-living infective larvae that will infect all NZSLs. These, in turn, would be the source of transmmary transmission for future generations of pups. Evidence to support this hypothesis was the successful culturing of ensheathed L_3s in the field and the finding of similar larvae (without a sheath) in the subcutaneous tissues of NZSLs (especially in pups, in an adult male and in non lactating females). The general size of these L_3 was consistent with each other and with reports of similar stages of *Uncinaria lucasi* in northern fur seals (Lyons, 1963; Lyons and Keyes, 1978). The finding of larvae in the subcutaneous tissue of NZSL female pups suggests these may be a source of transmammary infection for their offspring.

It is not known when pups expel hookworms from their intestines or what mechanisms are associated with this phenomenon. The immune response developed by the host towards parasites is part of a complex dynamic between the two parties, with the ideal outcome being a balanced relationship, and the two extremes resulting in disease or in the expulsion of parasites. Necropsy of NZSL yearlings has shown that they no longer harbour intestinal infection with hookworms.

One way to maintain the hookworm life cycle on the rookery is the possibility that larvae could overwinter in the sand. In the current study, such larvae were found in soil samples at the end of the breeding season but were not present in samples taken at the same sites at the beginning of the following season. However, this was not surprising as the sand at Sandy Bay Beach is washed away during the winter. This suggests that the only likely source of infection for future generations of pups on this rookery is the

hypobiotic larvae that reactivate during lactation. It is unknown whether all of these inhibited larvae reactivate at the next lactation or only some, implying that a single pool of dormant larvae would ensure that several generations of pups could become infected, without requiring reinfection. Studies in captive pinnipeds have shown that larvae could at least survive several years before being transmitted to the pup (Lyons and Bigg, 1983; Lyons and Keyes, 1984). Overall, the biology of *Uncinaria* spp. in NZSLs resembles that reported for *Uncinaria lucasi* in other pinniped hosts (Lyons et al., 2000a, 2000b).

Clinical pathology associated with hookworm infection in NZSL pups was similar to lesions reported in dogs infected with *Ancylostoma caninum* (Miller et al., 1971; Kalkofen, 1987). It mainly consisted of haemorrhagic enteritis with large amounts of frank blood in the intestinal lumen. However, contrary to the situation reported in dogs, there was no apparent relationship between burden and lesions in the NZSL. In addition, the present research did not succeed in showing any relationship between haematological parameters and the occurrence of hookworm infection. There was no evidence of anaemia in live infected pups. Why some pups succumbed and not others is at present uncertain.

Another possible pathological role of *Uncinaria* spp. in NZSLs was its effect on neonatal growth. Even though studies investigating the effect of parasitism on neonatal growth in wild animal species are very scarce, hookworm infection may be associated with weight loss, lack of appetite and growth delay in human neonates, children and in young domestic carnivores (Miller, 1971; Anderson, 2000; Kucik et al., 2004). In NZSL pups, hookworm infection would not impact on pup growth and survival (Chilvers et al., in preparation).

Some associations between pathogens happen to be synergistic: each species increases the pathogenicity of the other (Petney and Andrews, 1998). This may have been the case in NZSL pups infected with hookworms during the *Klebsiella pneumoniae* epidemics. Parasites could have debilitated pups, and these may have consequently become more susceptible to the bacterial pathogen.

II. *Klebsiella pneumoniae* epidemics

Klebsiella pneumoniae had not been identified as a cause of neonatal death prior to 2001/2002. During the 2001/2002 and 2002/2003 breeding seasons, this bacterium was cultured from a wide range of lesions and organs collected in NZSL pups at necropsy. In this respect, the high mortality events caused by *K. pneumoniae* during these two seasons fulfil the requirements to be described as an epidemic, in that there was a sudden onset and a high incidence of infection. Further evidence in support was the characterisation of *K. pneumoniae* as a single bacterial clone. Interestingly, it was different from *K. pneumoniae* cultured from adult NZSLs before and during the epidemics, although it was isolated from one adult male on the Otago Peninsula after the epidemics. The actual source of this particular clonal strain remains uncertain. From the limited testing undertaken, it was apparent that this *K. pneumoniae* clone was not resistant to common antimicrobial agents. This would suggest that it did not originate from humans as antibiotic resistance in human isolates of *Klebsiella* species is a common feature.

To investigate whether *K. pneumoniae* causing epidemics in NZSLs was a novel pathogen to the rookery, a serological survey was undertaken using WB. Results showed a high prevalence of anti-*Klebsiella* antibodies in adults, indicating that *Klebsiella* species were circulating in the NZSL population. In contrast, the seroprevalence in pups older than a month was only 16% regardless of whether it was an epidemic year or not, which suggested limited maternal transfer of passive immunity against *Klebsiella* pathogens. Even after the 2001/2002 and 2002/2003 epidemic seasons, there was no evidence of maternal transfer of anti-*Klebsiella* antibodies as these pups had a seroprevalence of 0%. This implies that young pups were not passively protected against *K. pneumoniae* infections. However, it was not possible to accurately determine the sensitivity of the WB test as NZSLs are endangered animals and experimental infections required to optimise test sensitivity were not possible. A limitation to these findings is also the lack of specificity of the WB. It was shown that this assay could not distinguish between two species of *Klebsiella* (*Klebsiella oxytoca*

and *K. pneumoniae*). Therefore this technique did not allow drawing a definite conclusion regarding the presence or the absence of this *K. pneumoniae* strain in the Sandy Bay Beach population prior to the epidemics.

Lesions associated with *K. pneumoniae* in NZSLs were dominated by arthitis, often in several joints, and septicaemia. A diverse range of lesions have been separately reported to be caused by *K. pneumoniae* in other animals, including humans (Giles et al., 1974; Jackson et al., 1980; Enurah et al., 1988; Coletti et al., 2001; Park et al., 2004). A feature of the disease in NZSLs was the broad spectrum of lesions that were caused by this pathogen and that were often observed in multiple organs of the same animal. Most *Klebsiella* spp. infections reported in other animals are generally not associated with a multi-systemic presentation of lesions.

III. Variability in the virulence of the opportunistic Klebsiella pneumoniae

Infections with opportunistic pathogens naturally occur in wild animals. Their functional immune system develops an adapted response and keeps the antigens in memory. Despite being specific, cellular immunity can protect individuals in the presence of slightly different pathogens. However, it is possible that a small genotypic difference between two strains of the same bacterial species generates a significant modification of its pathogenicity. It has been shown experimentally that the virulence of *K. pneumoniae* causing metritis in horses was directly proportional to the “degree of encapsulation” of the bacteria (Kikuchi et al., 1987). Even though antibodies against *K. pneumoniae* may cross-react with other Klebsiellae, it is likely that the presence of a particular type of capsular antigens requires highly specific antibodies. This hypothesis could explain the absence of immune protection of NZSL pups during the *K. pneumoniae* epidemics, despite the presence of general anti-*Klebsiella* antibodies at least in one in five pups. Highly virulent agents may owe their pathogenicity to the absence of highly specific immunity in the hosts.

IV. Emerging diseases and circulation of pathogens

Global environmental and nutritional stresses may challenge the health of marine mammals and consequently enhance the emergence of pathogens in the marine ecosystem, which may occasionally lead to disease outbreaks (Harwood and Hall, 1990; Harvell et al., 1999). Oceans may also serve as "incubators of human diseases" (Harvell et al., 1999). Indeed, emerging infectious diseases of wildlife in general represent a potential threat for the health of both humans and domestic animals. Many pathogens including the bacterial genera *Clostridium*, *Klebsiella*, *Legionella* and *Salmonella* are commonly found in estuaries and oceans (Grimes, 1991). In regions of the world where consumption of seals and polar bears by humans is a common practice, the zoonotic risk is even more important (Measures and Olson, 1999; Forbes, 2002). On the other hand, the development of ecotourism in remote latitudes raises the issue of human-associated pathogens being introduced into naïve wildlife populations. The code of conduct imposed to travel agents operating in Antarctica and in the Sub-Antarctic regions is very strict and so far is no evidence that this lucrative activity had led to bacterial pollution of such wild locations (Bonnedahl et al., 2005). There was no evidence that the epidemic strain of *Klebsiella pneumoniae* isolated from NZSLs on Enderby Island could have been introduced by tourists or scientists. Nevertheless, with the blossoming of the tourist industry in wild sanctuaries and natural reserves, the emergence of new diseases in breeding colonies of birds and pinnipeds has to be expected. This is another reason why continuous health monitoring in marine mammals is a key component for understanding emerging and endemic pathogens and their epidemiology.

V. Individual susceptibility to disease

It has been suggested that the impairment of cellular components of pinniped immune response by environmental factors could partly explain the resurgence of pathogens in wildlife and the mass mortalities affecting the marine ecosystem (Bleavins and Aulerich, 1983; Harwood and Hall, 1990; DeSwart et al., 1993; Harvell et al., 1999; Beckmen et al., 2003). In addition, the genetic diversity of some pinniped species has been reported to decline, as a direct consequence of decreased population sizes. In such

a small population as the NZSLs, the genetic diversity may be reduced. Inbreeding becomes inevitable in declining populations, leading to increased homozygosity for some genes, including the MHC genes. Reduced diversity of these genes has been suggested to be a factor in susceptibility to infectious diseases in wild animals, including otariids (O'Brien and Evermann, 1988; Acevedo-Whitehouse et al., 2003; Lento et al., 2003). It has been demonstrated that low genetic heterozygosity in California sea lions, and possibly in other pinniped species, was associated with loss of fitness (Acevedo-Whitehouse et al., 2006). Studies investigating the relationships between variability of MHC genes and the various causes of mortality in NZSL neonates presented in this research are in progress. It is likely these results will underpin the consequences of low genetic diversity in NZSLs and their individual susceptibility to disease (Lento et al., 2003). Such studies could provide, at least in part, some level of explanation for the variation of pathogenicity of hookworms in individual NZSL pups.

Conclusion of the Discussion

Working with an endangered species such as the NZSL, in a remote location, brings some constraints. These include the difficulty of obtaining research permits and the challenge to collect samples from wild animals, often in arduous field conditions.

Nevertheless, this particular body of research has revealed information about some aspects of neonatal mortality in NZSLs. As with any scientific study, there have been additional questions raised during this investigation and these should be addressed in future research on mortality in NZSL pups. Such studies should focus on reinforcing data collected on the life cycle of hookworms and faecal surveys should be extended to older pups in the season to clarify the persistence of the infection in pups. Health monitoring should be carried on at Sandy Bay Beach to ensure the continuity of data from this NZSL subpopulation, especially to monitor the role of *K.pneumoniae* in subsequent breeding seasons. Bacteriological analyses should be conducted on faeces of wild seabirds living on this rookery to determine if they play a role in the epidemiology of pathogens circulating in the breeding colony, including *Klebsiella* species.

Despite its status of being an endangered species protected by conservation laws, the NZSL population seems to be declining. The occurrence of epidemics at Sandy Bay Beach for the last decade (1997/1998, 2001/2002 and 2002/2003) has shown that NZSLs, especially pups, could be highly susceptible to some novel pathogens. The main natural causes of mortality (including trauma, bacterial and hookworm infections, starvation and stillbirth) are recurrent. In addition, epidemics involving bacterial pathogens also seem inevitable as they occurred in three of the last nine years. These mortality factors need to be given considerable importance when establishing management plans of an endangered species such as the NZSL.

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APPENDIX 2.1

Gross findings associated with stillbirth in New Zealand sea lion (NZSL) pups

Fig. 1. New Zealand sea lion female and its stillborn pup.
Pups start moving and calling after being freed from the foetal membranes by the mother, a few seconds only after birth. In the present case, the pup was never observed moving and breathing but the female stayed with its pup for the whole day.

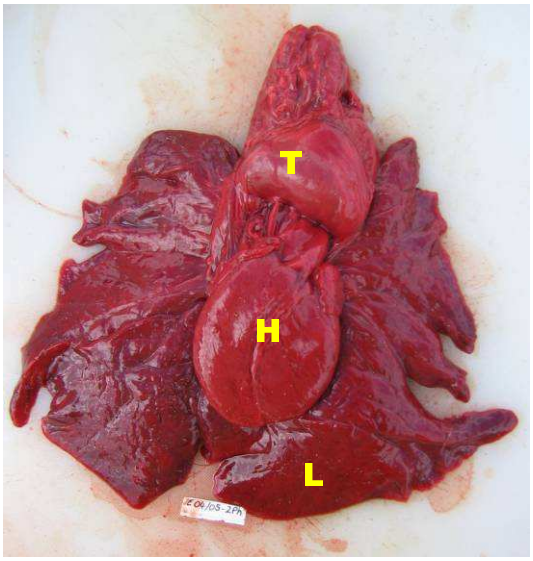

Fig. 2. Cardio-respiratory system of a stillborn pup. Lungs are characterized by a pink colour when they are functional (insufflated), unlike the dark and dense lungs of a stillborn pup. L: lungs; H: heart; T: thymus.

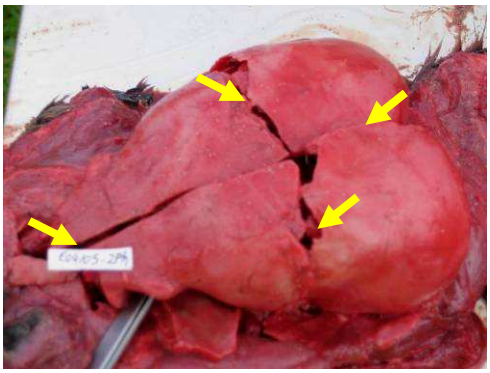

Fig. 3. Dorsal view of the dissected skull of a stillborn pup. Another characteristic of stillborn pups is the absence of connection between the skull pieces, as indicated by arrows.

APPENDIX 2.2

Histopathological findings associated with stillbirth in four NZSL pups

Histological findings	No. of pups with lesion
Respiratory system	
Atelectatic lungs	2
Amniotic fluid in airways	3
Presence of inflammatory cells in airways	1
Embolic pneumonia	2
Thymus	
Congestion	1
Liver	
Diffuse hepatocellular vacuolation (probable lipidosis)	1
Kidneys	
Severe diffuse haemorrhage	1
No gross lesions	3

APPENDIX 2.3

Histological findings associated with starvation/malnutrition in five NZSL pups

Histological findings	No. of pups with lesion
Respiratory system	
Diffuse congestion	1
Fluid/exsudate in airways	2
Acute suppurative pneumonia	1
Acute lobular haemorrhage	1
Acute interstitial oedema	1
Acute alveolar emphysema	1
Lymphatic tissues	
Oedematous lymph nodes	1
Very pale lymph nodes	1
Tonsil	
Reactive lymphoid follicles	1
Liver	
Mild diffuse congestion	1
Intrahepatic cholestasis	1
Kidneys	
Severe diffuse haemorrhage	1

APPENDIX 2.4

Summary of statistical outputs

for neonatal mortality in NZSLs

(Statistical computer package SAS, version 9.1, SAS Institute Inc., Cary, NC, USA)

- **Mortality rate from 1998/1999 to 2004/2005**

GENMOD Procedure
Class: sex, year
Model (logit, binomial, type 3 error): survival= sex, year, sex*year

LR Statistics For Type 3 Analysis

Source	DF	Chi-Square	Pr > ChiSq
sex	1	4.57	0.0325
year	6	162.94	<.0001
sex*year	6	0.94	0.9877

Least Squares Means (LSM)

Effect	year	Estimate	Standard Error	DF	Chi-Square	Pr > ChiSq
year	1998-1999	-2.6773	0.1800	1	221.32	<.0001
year	1999-2000	-2.3514	0.1578	1	222.12	<.0001
year	2000-2001	-2.1057	0.1356	1	241.12	<.0001
year	2001-2002	-0.7877	0.1075	1	53.74	<.0001
year	2002-2003	-1.2607	0.1090	1	133.73	<.0001
year	2003-2004	-1.8821	0.1311	1	205.97	<.0001
year	2004-2005	-1.9694	0.1453	1	183.80	<.0001

Transformation of LSM:

Year	logit LSM	Standard error(se)	z-value	lower bound	upper bound
1998-1999	-2.6773	0.1800	1.96	-3.0301	-2.3245
1999-2000	-2.3514	0.1578	1.96	-2.6606	-2.0421
2000-2001	-2.1057	0.1356	1.96	-2.3714	-1.8399
2001-2002	-0.7877	0.1075	1.96	-0.9984	-0.5770
2002-2003	-1.2607	0.1090	1.96	-1.4743	-1.0470
2003-2004	-1.8821	0.1311	1.96	-2.1390	-1.6251
2004-2005	-1.9694	0.1453	1.96	-2.2541	-1.6846

Year	Nominal survivors*	lower bound	upper bound	Nominal Dead pups
1998-1999	0.9357	0.9109	0.9539	0.0643
1999-2000	0.9130	0.8851	0.9347	0.0870
2000-2001	0.8915	0.8629	0.9146	0.1085
2001-2002	0.6873	0.6404	0.7307	0.3127
2002-2003	0.7791	0.7402	0.8137	0.2209
2003-2004	0.8679	0.8355	0.8946	0.1321
2004-2005	0.8775	0.8435	0.9050	0.1225

* logit LSM ± z*se

- **Causes of mortality from 1998/1999 to 2004/2005: effect of sex and year**

GENMOD Procedure
Class: sex, year
Model (logit, binomial, type 3 error): cause (stillborn or starvation or trauma or bacterial or hookworm infection)= sex, year, sex*year

1. STILLBORN

LR Statistics For Type 3 Analysis

Source	DF	Chi-Square	Pr > ChiSq
year	6	10.61	0.1012
sex	1	1.09	0.2967

2. STARVATION

LR Statistics For Type 3 Analysis

Source	DF	Chi-Square	Pr > ChiSq
year	6	29.51	<.0001
sex	1	3.45	0.0632
year*sex	6	11.33	0.0787

3. TRAUMA

LR Statistics For Type 3 Analysis

Source	DF	Chi-Square	Pr > ChiSq
year	6	5.18	0.5214
sex	1	4.46	0.0348
year*sex	6	14.59	0.0237

4. BACTERIAL INFECTION

LR Statistics For Type 3 Analysis

Source	DF	Chi-Square	Pr > ChiSq
year	6	43.14	<.0001
sex	1	7.24	0.0071
year*sex	6	9.15	0.1654

5. HOOKWORM INFECTION

LR Statistics For Type 3 Analysis

Source	DF	Chi Square	Pr > ChiSq
year	6	9.68	0.1390
sex	1	5.33	0.0210
year*sex	6	5.55	0.4759

- **Hookworm in NZSL pups: prevalence and burden**

1. General prevalence of hookworm infection at necropsy

GENMOD Procedure
Class: sex, year, body condition (bc), morphometric index (mor_index), time ('prev'=1 for prevalence of hookworm infection at necropsy before mid January and 'prev'=2 after mid January)
Model (logit, binomial, type 3 error): general prevalence of hookworm infection at necropsy (gen_hookw)= sex, year, bc, mor_index, prev

LR Statistics For Type 3 Analysis

Source	DF	Chi-Square	Pr > ChiSq
year	6	36.67	<.0001
sex	1	0.00	0.9987
bc	3	2.65	0.4496
mor_index	1	0.06	0.8044
prev	1	37.99	<.0001

2. Hookworm infection as a cause of mortality

GENMOD Procedure
Class: sex, year, bc, prev
Model (logit, binomial, type 3 error): hookworm (hookworm infection as primary cause of mortality)= year, sex, year*sex, bc, mor_index, prev

LR Statistics For Type 3 Analysis

Source	DF	Chi-Square	Pr > ChiSq
year	6	8.62	0.1961
sex	1	5.30	0.0214
year*sex	6	4.30	0.6361
bc	3	2.24	0.5242
mor_index	1	0.11	0.7432
prev	1	18.85	<.0001

3. Hookworm burden

MIXED Procedure
Class: sex, year, bc, bacterial infection diagnosed at necropsy (gen_bact)
Model log-transformed burden (logburden)= year, sex, bc, gen_bact

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
year	6	78	2.22	0.0494
sex	1	78	1.24	0.2698
bc	3	78	0.10	0.9583
gen_bact	1	78	0.05	0.8157

Transformation Least Square Means (LSM)-logburden to LSM-burden

Effect	year/sex/gen_bact	LSM	standard error	lower bound	upper bound
year	1998-1999	6.4	0.4	5.6	7.1
year	1999-2000	6.7	0.3	6.1	7.3
year	2000-2001	6.6	0.5	5.7	7.6
year	2001-2002	5.8	0.5	4.9	6.7
year	2002-2003	6.7	0.4	5.8	7.6
year	2003-2004	7.5	0.3	6.9	8.1
year	2004-2005	7.3	0.4	6.6	8.0
sex	1	6.6	0.2	6.2	7.0
sex	2	6.9	0.2	6.4	7.3
gen_bact	0	6.7	0.2	6.3	7.2
gen_bact	1	6.7	0.2	6.2	7.1

Hookworm burdens by year, sex and diagnosis of bacterial infection

Effect	Nominal mean burden	lower bound	upper bound
1998-1999	591.8	276.5	1266.7
1999-2000	790.4	440.1	1419.8
2000-2001	761.4	302.2	1918.6
2001-2002	333.9	135.9	820.2
2002-2003	825.8	345.0	1976.7
2003-2004	1781.3	954.9	3322.8
2004-2005	1439.3	718.3	2883.9
sex 1 (male)	709.2	481.4	1044.9
sex 2 (female)	950.8	619.5	1459.4
gen_bact 0 (no bact infect)	852.7	568.9	1278.1
Gen_bact 1 (bact infect)	790.8	504.9	1238.6

APPENDIX 2.5

Post-mortem examination of NZSL pups: special features for field necropsy at Sandy Bay Beach

1. General SOP

Standard procedure for post-mortem examination of marine mammals is as described by Duignan (1999). Detailed below are additional steps used on the Auckland Islands. Supplementary protocols for research on hookworm infection in NZSL pups are mentioned here but are further detailed in Appendices 5.1, 5.2, 5.3, 5.4 and 5.5.

2. Special features of field necropsy at Sandy Bay Beach (Enderby Island)

2.1. *Materials and wares*

- Staff: use disposable clothes or plastic yellow rain coat and trousers, gumboots exclusively used for post-mortem, plastic gloves of different sizes, sunscreen or rain-hat, sunglasses or ski goggles depending on the weather conditions.
- Post-mortem site and table:

  Clean area and check that the table is stable and horizontal.

  Place a bucket by the table to dispose of viscera and wastes during necropsy.

  Ensure the skuas (scavenging birds) do not have access to the bucket. Grab a few bamboo sticks to keep them away from carcass and waste bucket during necropsy.
- Record observations with pencils and weatherproof notebook.
- Scales: weight scale (Salter Spring scale ± 50grams, Sa;ter Housewares Ltd, Kent, UK); meter scale (clean before use)
- Use a various range of tools and containers to cut, measure, identify, sample and store (1.8ml cryovials, Viagen, Nunc©).

2.2. *Life history*

- Attribute a post-mortem number as followed: E04/05-23Ph (E stands for *Enderby*, 04/05 for *2004/2005 breeding season*, 23 for *the 23rd animal necropsied in the saison*, Ph for *Phocarctos hookeri*).

- Note all elements contributing to diagnosis (e.g. circumstances of death, time of death, weather prior to death, number of animals around carcass).

2.3. ***External examination***

- Note if the carcass has been scavenged and what organs are missing (abdominal or thoracic).
- Record sex, length, girth at umbilicus, weight (not relevant if carcass has been scavenged).
- Observe buccal mucosa, teeth and tongue. Check for the presence of sand, grass or milk in buccal cavity.
- Note all external wounds and scars (precise their location, size and depth and the presence of pus or blood).
- Note the presence of external parasites (lice) and collect some in 70% ethanol (for DNA analysis) and in 10% formalin (for morphological analysis).
- Carefully check the joints as arthritis was a typical lesion observed during the *Klebsiella pneumoniae* epidemics.
- Examine the umbilicus (is it fresh, infected or scavenged?).
- Note body condition and measure the ventral abdominal blubber depth.

Supplementary protocol for research on hookworm infection in NZSL pups:

For very fresh carcasses only (still warm), take a blubber sample of approximately 10 cm^2 over the full blubber depth, 2 cm posterior to the umbilicus. This sample will be later processed through a Baermann apparatus system (*see* Appendix 5.1).

2.4. ***Internal examination***

- Collect specimens from the following tissues into 10% formalin for histology: skin, skeletal muscle, brain, spinal cord, tongue, tonsil, thyroid, trachea, lungs, heart, lymph nodes, diaphragm, liver, spleen, pancreas, adrenal glands, kidney, stomach, intestine, urinary bladder, and gonads.
- Collect specimens from the following tissues into cryovials for bacteriology: liver, lung, tonsil, thymus, lymph nodes, spleen, faeces and any lesions or exudates.

- Check if all skull bones are connected.
- Examine both thoracic and peritoneal cavities for purulent discharge. In the presence of such fluid, take a bacterial swab.
- Check the floatability of lungs in a bucket filled with water (lungs that were never inflated will sink; this sign contributes to diagnose stillbirth). Sample the lungs at different sites (different lobes) for histology.
- Carefully examine the different cardiac chambers for congenital abnormalities.
- Check the integrity and morphology of the diaphragm (note visceral hernia or signs of trauma on the surface).
- Observe the gall bladder (a full gall bladder generally indicates that the pup had not had a recent meal).
- Describe the stomach and intestinal contents (e.g. milk, sand, grass, stones, water, frank blood).
- Collect about 20 hookworm specimens in ethanol.

Supplementary protocol for research on hookworm infection in NZSL pups:
Collect the intestinal content after rinsing the intestinal tract (see Appendix 5.3). Count the total number of hookworms to obtain the parasitic burden (see Appendix 5.4).

- Check the integrity of the distal intestinal portion (search for congenital abnormality *atresia ani*).
- At the end of the internal examination and once all samples for bacteriology and histology have been collected, open all joints (fore and hind limbs and atlanto-occipital joint) with a small and clean incision. In the presence of pus or abundant discharge, take a swab for bacteriology.

Supplementary protocol for research on hookworm infection in NZSL pups:
Collect an extra sample of faeces for parasitology analyses (the other one is for bacteriology) (*see* Appendix 5.5).

- Check that all samples (cryovials) are labelled.

3. Disposal of the carcass and viscera

- Place the carcass and all other tissue wastes in the bucket. If the pups was euthanized on human grounds, DO NOT EMPTY THE BUCKET ON THE BEACH but bury its content (scavenging birds are very sensitive to the drug used to euthanase pups).
- Otherwise, empty the bucket on the beach, skuas and giant petrels will quickly "clean" the scene.

4. Disinfection of necropsy tools and clothing

- This is a critical step as NZSL pups can be infected with highly pathogenic and potentially zoonotic bacteria. Place all the disposable scalpel blades and needles in the "sharp's" container.
- Gather all necropsy tools on a tray.
- Scrub the table with detergent (do not leave any blood stains in the corners). Rinse with water.
- Likewise scrub and rinse tools and clothes. Allow to dry in the sun or dry with paper towels.

5. Storing samples

- Bacteriology samples are stored in a liquid nitrogen bottle. Regularly check the seal of the bottle.
- Histopathology samples, parasites and DNA samples can be kept at ambient temperature.

6. Post-mortem report

Post-mortem observations should be typed as soon as possible.

Duignan *et al.* (1999) Unusual mortality of the New Zealand sea lion, *Phocarctos hookeri*, Auckland Islands, January–February 1998. Report of a workshop held 8–9 June 1998, Wellington, and a contingency plan for future events / compiled by Alan Baker. Wellington, NZ, Dept. of Conservation, 1999.

APPENDIX 3.1

First report and characterization of adult *Uncinaria* spp. in New Zealand Sea Lion (*Phocarctos hookeri*) pups from the Auckland Islands, New Zealand

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ORIGINAL PAPER

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Abstract Two species of hookworms (*Uncinaria lucasi* and *Uncinaria hamiltoni*) have been formally described from pinnipeds, but dissimilar types are noted from these hosts. This report is the first description of hookworms (*Uncinaria* spp.) from the New Zealand sea lion, *Phocarctos hookeri*. The nematodes were collected from dead pups on Enderby Island (Auckland Islands, 50°30', 166°17') during January and February, 2004. Standard measurements of male and female hookworms were obtained, providing a general morphometric characterization of the hookworm species in *P. hookeri*. Considerable variations in the body length of adult hookworms were noted within the same host. The arrangement of some of the bursal rays differs from that described for *U. lucasi* and *U. hamiltoni*.

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Introduction

The New Zealand Sea Lion (NZSL), *Phocarctos hookeri* (Hooker's sea lion), is one of the rarest and most locally endemic members of the Otariidae. This species has been classified as "vulnerable" and "endangered" because of its small population size and limited distribution on the sub-antarctic Auckland Islands (Gales and Fletcher 1999). Studies conducted during the past 7 years on Enderby Island (Auckland Islands) have shown that hookworm infection is a significant cause of mortality in pups (Castinel et al. 2004).

Only two hookworm species, *Uncinaria lucasi* Stiles and *Uncinaria hamiltoni* Baylis, have been described from otariids (Stiles 1901; Baylis 1933, 1947). However, individual hookworms with intermediate morphotypes have been reported from pinniped hosts (Baylis 1947; Olsen 1952; Dailey and Hill 1970; Nadler et al. 2000), suggesting that additional biodiversity may be present but, as yet, uncharacterized. The present study provides a morphometric description of hookworm specimens collected from infected NZSL pups on Enderby Island. This description is necessary for more comprehensive comparisons of *Uncinaria* spp from various pinniped hosts.

Materials and methods

Necropsies to investigate neonatal mortality were conducted in the field on NZSL pups found dead on Sandy Bay beach, Enderby Island (Auckland Islands, 50°30'; 166°17') during the period from January 7 to February 14 2004. Mature adult hookworms were collected from the intestinal tracts of 4- to 7-week-old pups and stored in 100% ethanol. Thirty mature specimens were selected from four different pups—in which individual burden ranged from 2,310 to 7,080 parasites—for morphological study, cleared with a few drops of lactophenol and mounted temporarily on slides. The hookworms were examined using a light microscope, and images were acquired using a digital camera. Morphological study concentrated on features used pre-

viously to characterize *U. lucasi* and *U. hamiltoni*, including the buccal capsule, esophagus, male copulatory bursa, and female tail. Fertile eggs (eight-cell stage) were obtained and measured by dissection of the uteri of females. Esophageal length was measured from the beginning of the esophageal tube (at the base of the buccal capsule) to the junction with the intestine; this differs from the variable measured by Baylis (1933) and Olsen (1952). In addition, some specimens of both genders were prepared for scanning electron microscopy (SEM) following standard procedures. Eight adult males and 11 adult females were measured using a microscope equipped with an ocular micrometer (Table 1). Measurements (in text) are means followed by standard errors (ranges are provided in Table 1). Ratios of some features were used to compare measurements between males and females. Analyses consisted of descriptive statistics and one-way analyses of variance (ANOVAs) to investigate the effect of gender on the measured variables. Adult *Uncinaria* spp specimens of each sex were deposited as vouchers in the US National

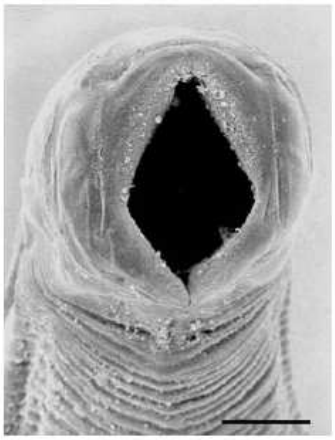

Fig. 1 SEM of the anterior end of an adult hookworm (female) from a NZSL pup in en face view. Bar scale: 40 μm

Table 1 Measurements[a] of *Uncinaria* spp. from NZSL pups, *U. hamiltoni* from *Otaria byronia* (Baylis 1933), *U. lucasi* from *C. ursinus* (Baylis 1947) and *U. hamiltoni* from *Otaria flavescens* (Berón-Vera et al. 2004)

	Uncinaria spp. from *Phocarctos hookeri* (present study)		*Uncinaria hamiltoni* from *Otaria byronia* (Baylis 1933)		*Uncinaria lucasi* from *Callorhinus ursinus* (Baylis 1947)		*Uncinaria hamiltoni* from *Otaria flavescens* (Berón-Vera et al. 2004)	
	Females	Males	Females	Males	Females	Males	Females	Males
Sample size	11	8	3	?	?	?	30	29
Body length	10.35±56 7.20–12.30	6.82±0.49 5.00–9.20	12.5–17.5	8.5–12.0	12.4–16.0	7.4–8.7	11.37±2.94 5.36–17.2	7.85±1.52 4.88–10.64
Buccal capsule length	0.23±004 0.22–0.25	0.20±0.004 0.19–0.21	0.32–0.38	0.28–0.30	0.24–0.28	0.21–0.24	0.28±0.03 0.21–0.34	0.24±0.02 0.21–0.28
Buccal capsule width	0.19±0.007 0.16–0.23	0.17±0.005 0.15–0.19	–	–	–	–	0.24±0.02 0.20–0.31	0.19±0.06 0.14–0.24
Teeth height	0.044±0.007 0.037–0.050	0.030±0.002 0.025–0.035	–	–	–	–	–	–
Esophagus length	1.07±0.021 0.99–1.16	0.91±0.021 0.86–0.99	1.60–1.90	1.50–1.55	1.25–1.40	1.10–1.20	1.15±0.11 0.82–1.34	1.09±0.19 0.79–1.83
Diameter of esophageal bulb	0.18±0.003 0.17–0.20	0.16±0.004 0.14–0.18	–	–	–	–	–	–
Nerve ring from anterior end of body	0.69±0.020 0.56–0.78	0.52±0.002 0.37–0.74	0.60–1.10	–	0.57–0.62	–	–	–
Spicule length	–	0.69±0.016 0.62–0.74	–	1.000	–	500–560	–	0.89±0.09 0.57–1.05
Accessory piece length	–	0.060–0.080	–	0.12–0.14	–	80–90	–	–
Vulva to posterior end	4.01±0.16 3.19–4.84	–	5.10–7.00	–	5.20–6.30	–	4.33±1.34 2.13–6.62	–
Length of tail	0.20±0.007 0.16–0.23	–	0.16–0.25	–	0.21–0.25	–	–	–
Egg length	0.13–0.14	–	0.135–0.138	–	0.12–0.14	–	0.10–0.14	–
Egg width	0.072–0.081	–	0.085–0.093	–	0.080–0.088	–	0.04–0.10	–

[a]Measurements are given in millimeters; they are all ranges with additional means/standard deviations for NZSL hookworms

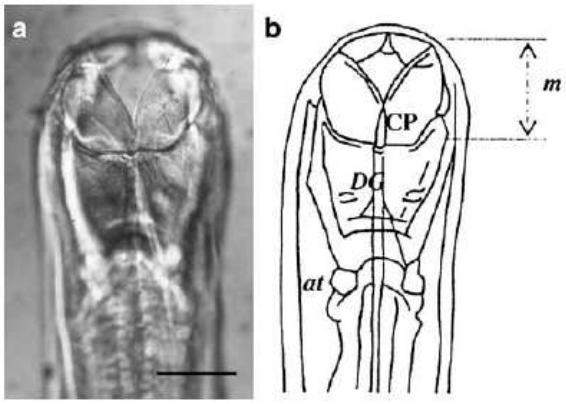

Fig. 2 Microphotograph (**a**) and drawing (**b**) of the buccal capsule of an adult hookworm (male) from a NZSL pup. Superficial dorso-ventral view showing the mouth opening (*m*), cutting plates (*CP*), dorsal gutter of esophagus (*DG*), and annular thickening (*at*) at the base of buccal capsule. Bar scale: 80 μm

Parasite Collection (USNPC No. 96483) (Agricultural Research Service, US Department of Agriculture, Beltsville, MD, USA).

Results

All hookworm specimens presented the general characteristics of *Uncinaria* spp. (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9), with the typical anterior bend resulting in a dorsal buccal aperture (Fig. 1). The mouth opening spans about one half of the buccal capsule depth in dorso-ventral view. There are two pairs of cutting plates, one anterior and one posterior (Figs. 2b and 4b), the latter almost entirely visible through the mouth opening in dorso-ventral view (Lyons and

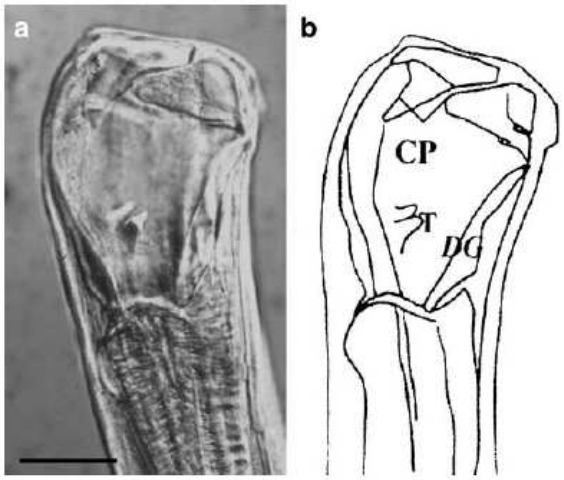

Fig. 4 Microphotograph (**a**) and drawing (**b**) of the buccal capsule of an adult hookworm (male) from a NZSL pup. Lateral view with the pair of teeth (*T*), cutting plates (*CP*), and dorsal gutter (*DG*). Bar scale: 80 μm

Delong 2005). *Buccal capsule:* The buccal capsule is elongated; 202.0±2.0 μm long by 167.5±5.3 μm wide in males and 232.5±3.7 μm by 195.4±6.6 μm in females. There is a pair of very well-developed sub-ventral teeth (Figs. 3b and 4b), with lengths from 25 to 35 μm in males and 27 to 50 μm in females. There is an annular thickening of the wall at the base of the buccal capsule (Fig. 2b), and the dorsal gutter of the esophageal gland is pronounced (Figs. 2b and 4b). Cephalic sensilla visible on the cuticle include two pairs of papillae and one pair of amphids surrounding the border of the mouth opening.

Esophagus: The esophagus is flask-shaped, common to the other species of *Uncinaria*. The length (measured

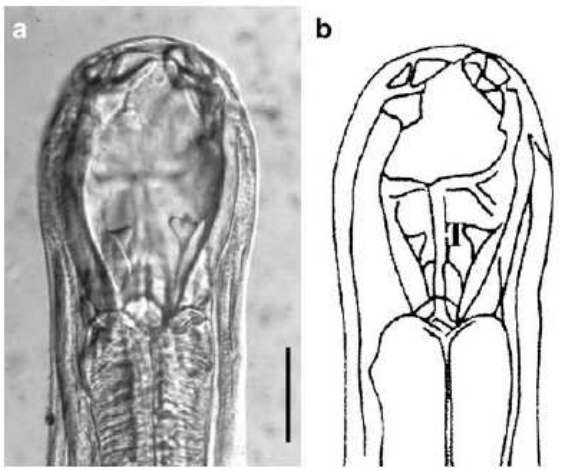

Fig. 3 Microphotograph (**a**) and drawing (**b**) of the buccal capsule of an adult hookworm (male) from a NZSL pup. Deeper dorso-ventral view underlining the pair of sub-ventral teeth (*T*). Bar scale: 80 μm

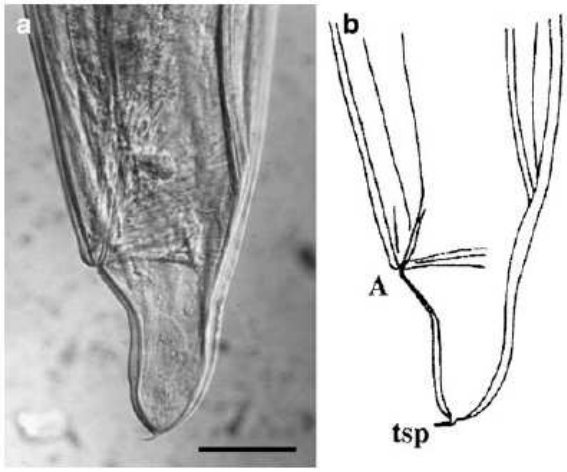

Fig. 5 Microphotograph (**a**) and drawing (**b**) of the terminal end of a female adult hookworm from a NZSL pup. Lateral view of the tail showing the anus (*A*) and the mucro or terminal spike (*tsp*). Bar scale: 80 μm

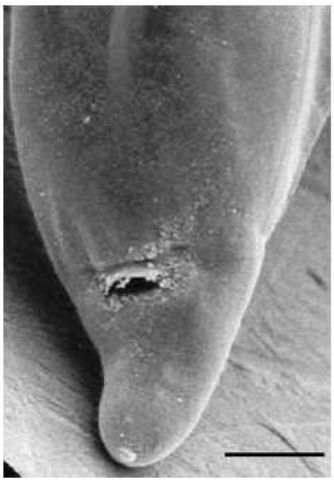

Fig. 6 SEM of ventral view of the tail of a female adult hookworm showing the anus. Bar scale: 50 μm

from anterior to posterior end) is 912.2±21.1 μm in males and 1,074.7±20.7 μm in females; the width at the base is 157.5±4.1 μm in males and 183.8±2.9 μm in females. The nerve ring surrounding the esophagus above the bulb region is at 526.2±70.4 μm from the anterior end of the body in males and at 685.5±22.5 μm in females.

Males: The males are 6.82±0.49 mm long. The two copulatory spicules are equal in length (688.5±16.3 μm). The gubernaculum is 60 to 80 μm long, oblong, and widens posteriorly. The bursa (Fig. 7a) shares similar features with that of order Strongylida (Olsen 1974; Schmidt and Roberts 1989). The ventral rays are fused and measure from one half to two thirds of the medio-lateral ray (Fig. 8b). The ventral rays diverge perpendicularly from the antero-lateral-ray axis (Fig. 8b). The tips of the postero- and antero-lateral rays are directed away from the medio-lateral ray. The antero-lateral ray does not touch the edge of the lateral lobe. The dorsal ray bifurcates distally with three small branches at the tips, and diverges from the outer ray. The dorsal lobe is very small and semicircular. The externo-dorsal ray is long, reaching the margin of the lateral lobe, and diverges proximally from the dorsal ray. Paired caudal papillae are present on the lateral lobe.

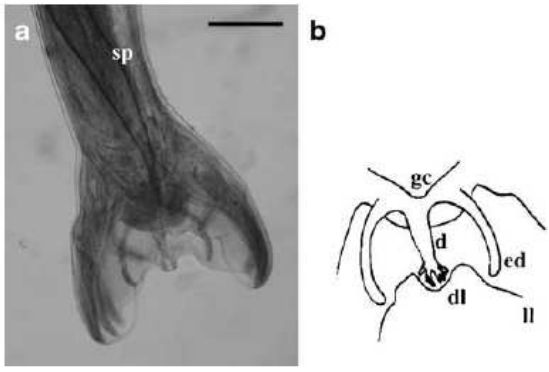

Fig. 7 Microphotograph (**a**) and drawing (**b**) of the terminal end of a male hookworm from a NZSL pup. Dorso-ventral view of bursa showing paired spicules (*sp*), genital cone (*gc*), dorsal (*dl*) and lateral (*ll*) lobes, and externo-dorsal (*ed*) and dorsal (*d*) rays. Bar scale: 150 μm

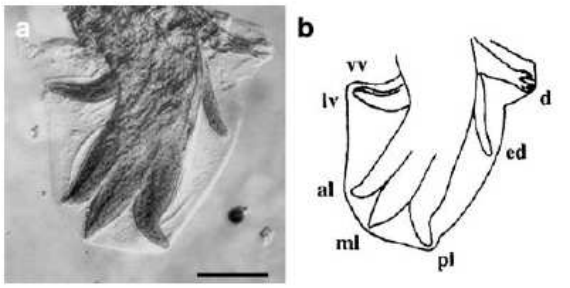

Fig. 8 Microphotograph (**a**) and drawing (**b**) of the terminal end of a male hookworm from a NZSL pup. Lateral view of copulatory bursa presenting the arrangement of ventro-ventral (*vv*), latero-ventral (*lv*), antero-lateral (*al*), medio-lateral (*ml*), postero-lateral (*pl*), externo-dorsal (*ed*), and dorsal (*d*) rays. Bar scale: 100 μm

Females: The females are 10.35±0.56 mm long. The vulva is prominent, and is located 4.01±0.16 mm from posterior end. The vagina is short and wide. The ovejectors are longitudinal and symmetrical, with a combined length of 450 μm. The tail (anal opening to tail tip) is 196.2± 6.8 μm long (Fig. 6). The tail mucro (Fig. 5b) is usually bent, and is between 10 and 30 μm long. A pair of caudal papillae is found at 38–40 μm from the terminal tip. The eggs are 136.6±0.7 μm long by 77.1±1.4 μm wide.

Results of statistical analyses are presented in Table 2 for both crude variables and ratios of the same parameters over the body length. There was a significant difference between males and females in body length ($p<0.001$), buccal capsule length ($p<0.001$) and width ($p<0.001$), esophageal length ($p<0.001$), the diameter of the esophageal bulb ($p<0.001$), and the distance of the nerve ring from the anterior end ($p<0.001$). Tests comparing the ratios of these variables to the total body length showed that gender had no effect on the position of the nerve ring ($p=0.63$); although increasing towards the level of statistical significance, all other p values for these size characters still support differences between males and females. This suggests that ratios related to the body length could correct the bias caused by the variations of size in the specimens examined.

Discussion

The measurements of *Uncinaria* spp. from NZSL pups showed a strong sexual dimorphism (body length, buccal capsule length, and esophageal length), but there were some morphological features of the buccal capsule and the esophagus that did not differ between sexes. Likewise, gender had no effect on the position of the nerve ring when statistical comparison included the ratio of the distance as based on total body length. This indicates that the ratios of

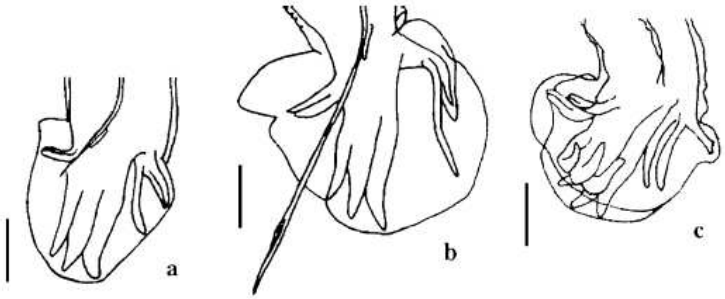

Fig. 9 Lateral views of male copulatory bursae from *Uncinaria* spp (redrawn): **a** *U. hamiltoni* from Baylis (1933), **b***U. lucasi* from Baylis (1947), **c***U. hamiltoni platensis* from Botto and Mañe-Garzon (1975). Bar scale: 200 μm

certain measured variables may provide more accurate conclusions than those conclusions based on specific measurements, especially when comparing *Uncinaria* spp. in pinniped hosts (George-Nascimento et al. 1992) where sexually mature hookworms can vary substantially in total body length. Ranges of body length in *Uncinaria* spp. from NZSL were similar to those from Berón-Vera et al. (2004), but were reduced relative to *U. lucasi* and *U. hamiltoni* as described by Baylis (1933, 1947). Almost all variables measured in hookworms from the southern hemisphere (Botto and Mañé-Garzón 1975; Berón-Vera et al. 2004) present a broad range with regard to measurements important for comparing species: buccal capsule length, esophagus length, distance of vulva to posterior end, spicule length, and egg dimensions (Table 1). Disparity in the size of mature hookworms within the same host was observed during the present study. The measurements from *Uncinaria* spp. found in the Northern Hemisphere show less variation (Nadler et al. 2000). Lyons (2005) quotes Olsen (1952): "While the body size of the hookworms from the sea lions tends to be larger than those from the fur seals, the relative proportions on a percentage basis remain almost the same." This pertains to *U. lucasi*, from northern fur seal (*Callorhinus ursinus*) and Steller sea lion (*Eumetopias jubatus*) pups from St. Paul Island, Pribilof Islands, Alaska (Olsen 1952). Using ratios may permit compensation for this type of bias. It seems inappropriate to include body width in comparisons for *Uncinaria* spp. in NZSL, not only because mounting specimens between a slide and a cover slip is highly likely to deform the diameter of a nematode, but because previous authors do not identify the precise site of body width measurement.

The arrangement of the bursal rays of *Uncinaria* spp in the NZSLs is characterized by the configuration of lateral rays resembling *U. lucasi*, and the externo-dorsal ray being much longer than the dorsal one is a feature more similar to *U. hamiltoni*. This reinforces the need for an extensive morphometric examination of *Uncinaria* species in pinnipeds.

Table 2 Effect of gender on measurements of *Uncinaria* spp. from NZSL pups

Variables	Difference between sexes (p value) for crude variables	Ratio to body length		Difference between sexes (p value) for ratio to body length
		Male	Female	
Body length	0.00015	–	–	–
Buccal capsule length	0.0000013	0.020–0.033	0.019–0.033	0.0212
Buccal capsule width	0.00657	0.017–0.032	0.017–0.022	0.000576
Esophagus length	0.000361	0.097–0.173	0.084–0.151	0.00417
Diameter of esophageal bulb	0.0000673	0.027–0.030	0.014–0.023	0.00920
Nerve ring to anterior end of body	0.015	0.060–0.088	0.053–0.096	0.6259
Spicule length	–	0.077–0.113	–	–
Vulva to posterior end of body	–	–	0.346–0.442	–
Tail	–	–	0.014–0.026	–

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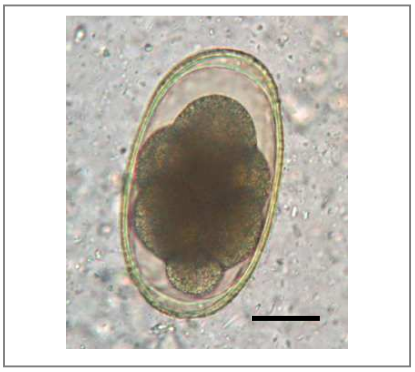
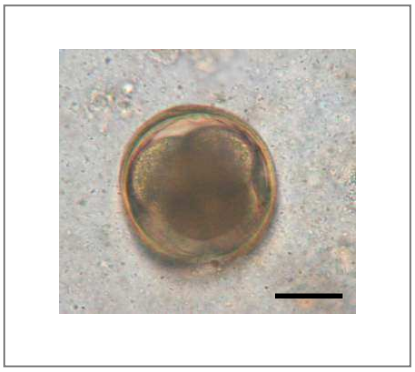
APPENDIX 3.2

Developmental stages of *Uncinaria* spp. from the New Zealand sea lion, *Phocarctos hookeri*

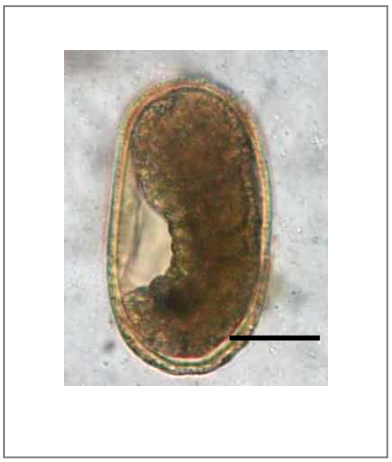
A.1



A.2

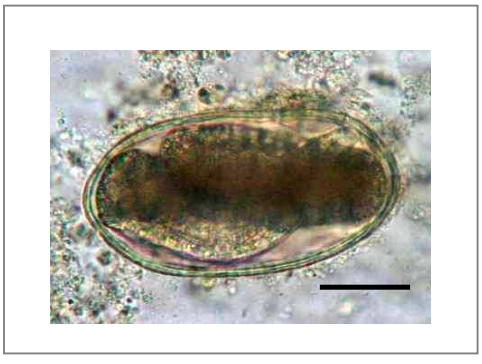


B.1



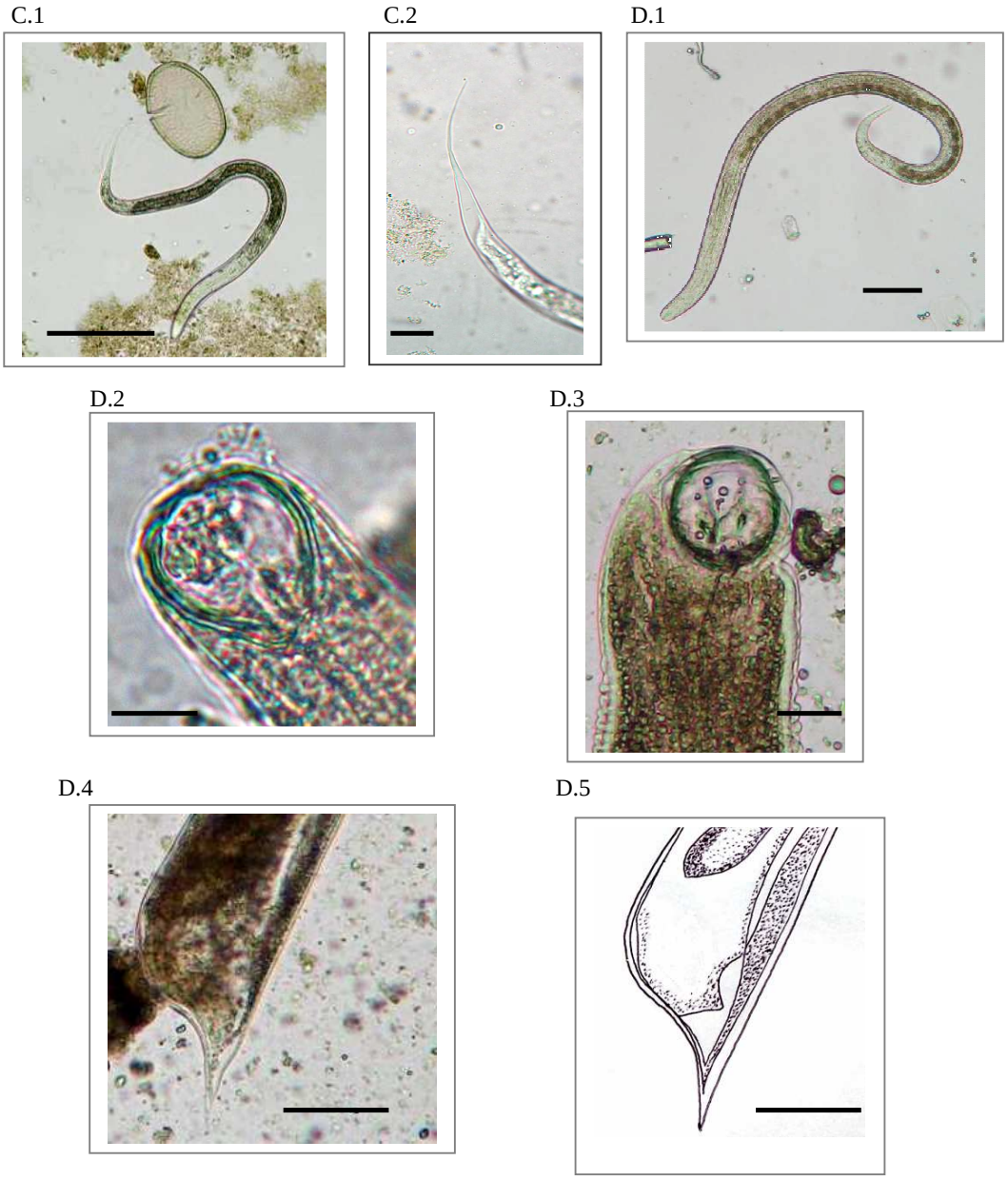
B.2

B.3



Figures A.1, A.2: *Uncinaria* spp. eggs from New Zealand sea lion pups. Bar scale 30μm

Figures B.1, B.2, B.3: larval stages of *Uncinaria* spp. inside egg. Bar scale 40μm



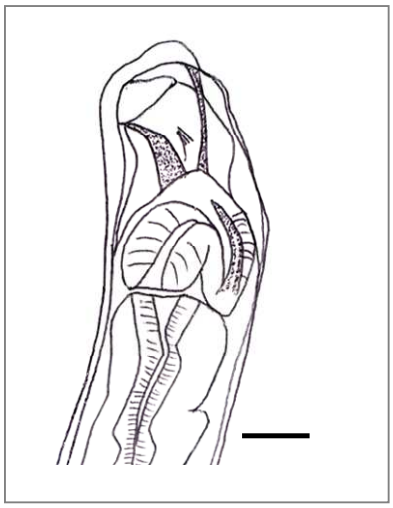
Figures C1: Free-living third-stage hookworm larvae (*in vitro* hatching) (C1). Hatching larvae are ensheathed (C.2). Bar scale 150μm.

Figures D.1, D.2: Hookworm larvae recovered from the stomach content of a New Zealand sea lion pup at one day (D.1) and 5 day old (buccal capsule, en-face view, D.2). Bar scales: 100μm (D.1) and 30μm (D.2).

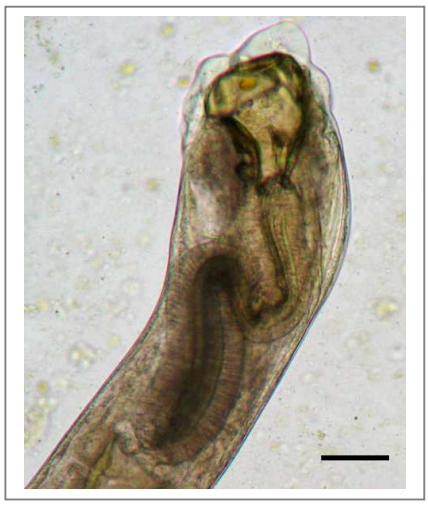
Figures D.3, D.4, D.5: Pre-adult hookworm found in the small intestine of a New Zealand sea lion pup at necropsy. D.3: buccal capsule (bar scale 40μm). D.4 and D.5: terminal end of a male specimen with copulatory bursa in formation (bar scale 80 μm).

E.1

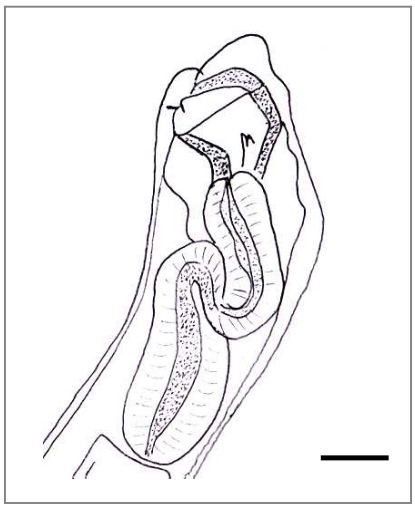
E.2



E.3



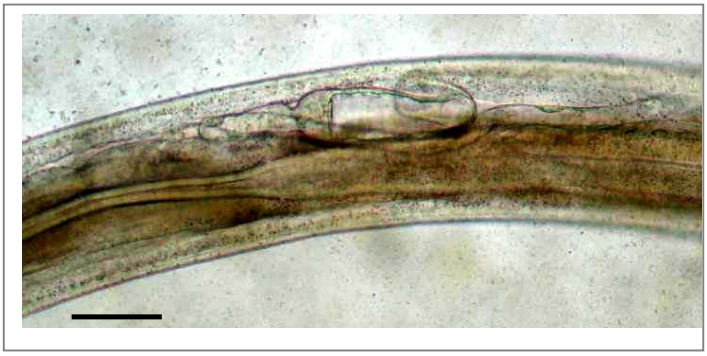
E.4



Figures E.1-E.4: lateral views of the anterior end of two pre-adult hookworms from New Zealand sea lion pups. Both specimens show a flexion of the oesophagus, which is not observed in the adult form. This feature was never reported in other *Uncinaria* species. The buccal capsule presents the characteristics of mature hookworms (e.g. teeth, cutting plates). Bar scale 100 μm.

E.5

E.6



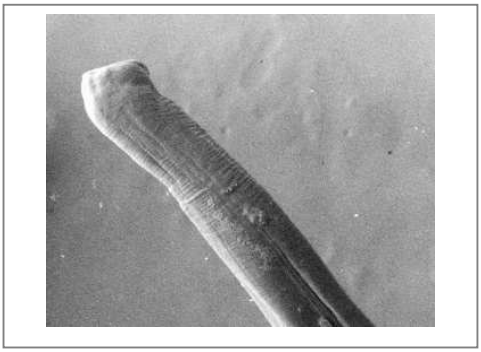
E.7

Figure E.5: Lateral view of a female hookworm collected in the small intestine of a New Zealand sea lion pup. The microphotograph is centred on the vulva. Bar scale 80μm.

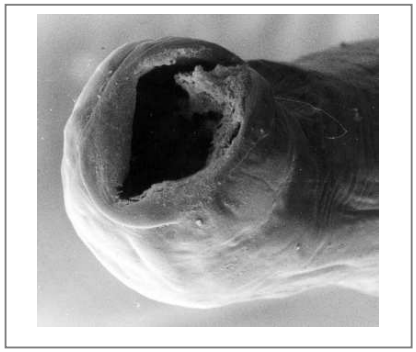
Figure E.6: Lateral view of the genital tract of the same immature hookworm female as in Figure E.5. The immature stage is illustrated here by the absence of eggs in the uterus. Bar scale 80μm.

Figure E.7: Lateral view of the terminal end of the same immature hookworm female as in Figure E.5. The typical morphological characteristics of the female hookworm are presented here, with the tail and terminal spike or mucro. Bar scale 100μm.

F.1



F.2



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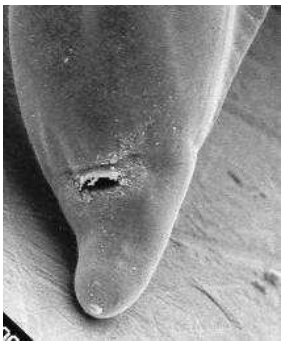
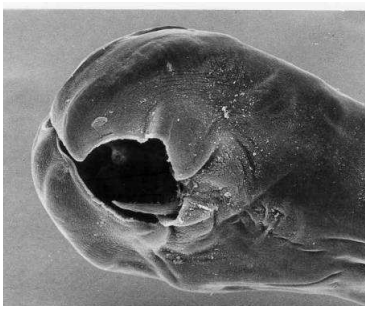
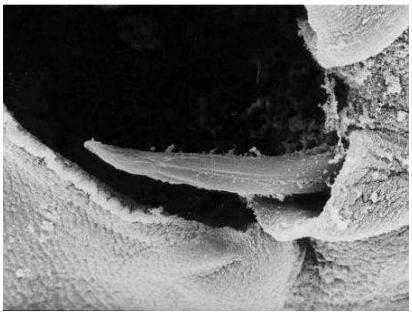


F.4

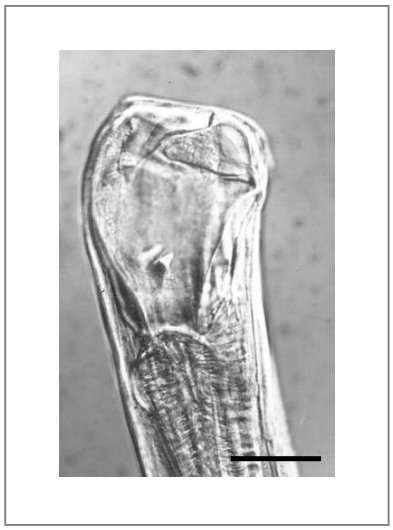


F.5

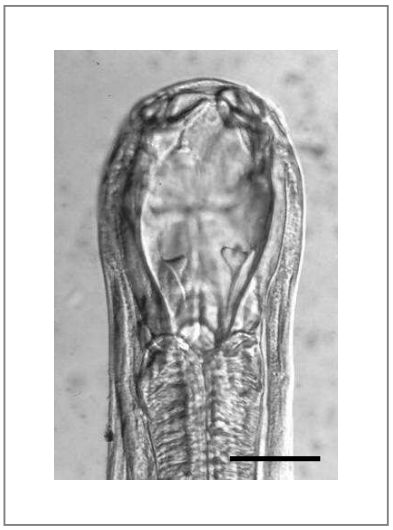


Figures F.1-F.5: Scanning Electron Microscopy of adult *Uncinaria* spp. from New Zealand sea lion pups. F.1: Anterior end of a female adult showing dorsal bending of the cephalic region, characteristic of the taxonomic Family (bar scale μm). F.2: Buccal opening of *Uncinaria* spp. from New Zealand sea lions (bar scale μm). F.3: Caudal end of a female adult hookworm from New Zealand sea lions, harbouring the anal pore and the terminal spike or mucro (bar scale μm). F.4, F.5: Caudal end of a male adult hookworm revealing the copulatory bursa (F.4) with one of the two spicules (F.5) emerging from the bursa (bar scale μm).

G.1



G.2



G.3

G.4

Figures G.1, G.2: Microphotographs of an adult hookworm from a New Zealand sea lion pup. G.1: Lateral view of the buccal capsule showing the pair of teeth and cutting plates. G.2: Sagittal view of the buccal capsule underlining the dorsal gutter in the background, between the teeth. Bar scale 80μm.

Figures G.3, G.4: Microphotographs of a male adult hookworm from a New Zealand sea lion pup. Dorsoventral views of bursa showing the paired spicules retracted (G.3) and externalized (G.4). Bar scale 150μm.

APPENDIX 4.1

Temperature records* - Laboratory, Enderby Island

Daily minima-maxima from 7.12.2004 to 19.1.2005

Date	Min (ºC)	Max (ºC)
7/12/2004	6.22	14.85
8/12/2004	6.22	14.47
9/12/2004	4.15	10.21
10/12/2004	4.15	14.85
11/12/2004	4.15	12.55
12/12/2004	3.74	11.38
13/12/2004	4.57	15.23
14/12/2004	4.99	17.14
15/12/2004	8.23	15.62
16/12/2004	7.03	17.52
17/12/2004	7.03	16.38
18/12/2004	6.22	16.76
19/12/2004	7.83	17.14
20/12/2004	7.43	16.38
21/12/2004	8.63	15.62
22/12/2004	8.63	16.38
23/12/2004	9.42	16.76
24/12/2004	8.63	15.62
25/12/2004	8.23	14.47
26/12/2004	9.42	16.76
27/12/2004	7.43	16.76
28/12/2004	10.21	17.9
29/12/2004	11.38	15.62
30/12/2004	6.62	13.7
31/12/2004	7.83	14.47
1/01/2005	7.83	16.76
2/01/2005	9.82	16.38
3/01/2005	9.42	16.76
4/01/2005	9.42	16.33
5/01/2005	9.03	16.76
6/01/2005	8.63	16
7/01/2005	9.82	17.9
8/01/2005	10.6	17.52
9/01/2005	8.63	19.42
10/01/2005	10.6	15.62
11/01/2005	8.23	15.62
12/01/2005	10.6	15.62
13/01/2005	10.99	17.52
14/01/2005	11.77	15.23
15/01/2005	10.99	19.04
16/01/2005	10.6	14.85
17/01/2005	9.82	17.14
18/01/2005	7.83	13.7
19/01/2005	6.62	17.9

*Hobo H8 datalogger, Scott Technical Instruments, Hamilton, New Zealand

APPENDIX 4.2

Temperature records* 2003/2004- Creek and hut sites, Sandy Bay Beach

Daily minima-maxima from 7.12.2004 to 23.1.2005

Creek site

Date	Min (ºC)	Max (ºC)
12/01/2004	11.38	12.93
13/01/2004	11.77	13.32
14/01/2004	12.16	13.32
15/01/2004	11.38	12.93
16/01/2004	10.60	13.32
17/01/2004	12.16	16.38
18/01/2004	11.77	12.93
19/01/2004	10.99	12.55
20/01/2004	10.21	12.93
21/01/2004	11.38	13.32
22/01/2004	12.16	14.09
23/01/2004	12.93	14.85
24/01/2004	12.55	14.09
25/01/2004	11.77	14.47
26/01/2004	12.16	14.09
27/01/2004	11.77	12.93
28/01/2004	11.38	12.93
29/01/2004	11.77	12.93
30/01/2004	11.77	13.7
31/01/2004	12.16	13.32
1/02/2004	11.77	12.93
2/02/2004	11.77	12.55
3/02/2004	11.38	12.55
4/02/2004	11.77	12.55
5/02/2004	10.6	13.32
6/02/2004	12.16	12.93
7/02/2004	10.99	12.93
8/02/2004	10.21	12.55
9/02/2004	11.38	12.16
10/02/2004	9.82	11.38
11/02/2004	10.6	11.38

* Hobo H8 datalogger, Scott Technical Instruments, Hamilton, New Zealand

Hut site

Date	Min (ºC)	Max (ºC)
12/01/2004	10.99	14.85
13/01/2004	11.77	15.62
14/01/2004	12.16	14.47
15/01/2004	10.99	14.09
16/01/2004	10.6	16
17/01/2004	12.55	14.85
18/01/2004	12.16	14.09
19/01/2004	10.6	14.85
20/01/2004	10.99	14.85
21/01/2004	11.77	15.23
22/01/2004	12.55	16
23/01/2004	12.93	16.76
24/01/2004	12.93	14.47
25/01/2004	11.77	16.76
26/01/2004	12.55	14.47
27/01/2004	12.16	13.32
28/01/2004	11.77	13.7
29/01/2004	11.77	14.09
30/01/2004	11.77	14.85
31/01/2004	12.16	14.09
1/02/2004	11.77	13.32
2/02/2004	12.55	11.38
3/02/2004	11.38	12.93
4/02/2004	11.38	12.93
5/02/2004	10.21	13.7
6/02/2004	12.16	13.32
7/02/2004	10.99	12.93
8/02/2004	10.21	12.93
9/02/2004	11.38	12.55
10/02/2004	9.82	11.77
11/02/2004	10.6	12.16
12/02/2004	9.42	11.38
13/02/2004	8.63	10.60
14/02/2004	9.82	10.99
15/02/2004	10.6	11.38
16/02/2004	10.99	11.77
17/02/2004	10.6	11.77
18/02/2004	10.21	10.99
19/02/2004	9.82	10.99
20/02/2004	9.42	10.60

APPENDIX 4.3

Temperature records* 2004/2005- Creek site, Sandy Bay Beach

Daily minima-maxima from 7.12.2004 to 23.1.2005

Date	Min (ºC)	Max (ºC)
7/12/2004	9.42	11.38
8/12/2004	9.42	9.82
9/12/2004	7.83	9.42
10/12/2004	7.03	8.23
11/12/2004	6.62	7.83
12/12/2004	6.62	8.23
13/12/2004	7.03	8.23
14/12/2004	7.03	9.03
15/12/2004	8.23	10.99
16/12/2004	8.63	10.6
17/12/2004	8.63	10.21
18/12/2004	7.83	9.42
19/12/2004	8.63	11.77
20/12/2004	9.42	11.38
21/12/2004	10.21	12.16
22/12/2004	10.21	11.77
23/12/2004	10.21	11.77
24/12/2004	10.21	11.38
25/12/2004	9.82	10.99
26/12/2004	9.82	10.6
27/12/2004	9.03	12.6
28/12/2004	10.99	11.77
29/12/2004	10.6	11.77
30/12/2004	9.82	11.38
31/12/2004	11.38	12.93

Date	Min (ºC)	Max (ºC)
1/01/2005	10.99	13.32
2/01/2005	12.16	13.32
3/01/2005	11.77	12.93
4/01/2005	11.38	12.55
5/01/2005	10.6	11.77
6/01/2005	10.99	12.93
7/01/2005	11.77	13.32
8/01/2005	12.16	13.32
9/01/2005	11.38	12.55
10/01/2005	11.77	12.55
11/01/2005	10.99	12.16
12/01/2005	11.38	12.55
13/01/2005	10.99	13.32
14/01/2005	11.77	12.93
15/01/2005	10.99	14.09
16/01/2005	10.99	13.32
17/01/2005	10.6	13.7
18/01/2005	10.21	13.32
19/01/2005	8.63	12.55
20/01/2005	10.99	13.32
21/01/2005	10.21	14.47
22/01/2005	10.21	13.7
23/01/2005	10.6	13.32

*Hobo H8 datalogger, Scott Technical Instruments, Hamilton, New Zealand

APPENDIX 5.1

SOP for recovering hookworm larvae from fresh tissue and milk samples from NZSL pups

Principle

Infective hookworm larvae may be found in subcutaneous tissues of NZSLs following percutaneous infection. In lactating females, these larvae are likely to migrate to the mammary glands and to be passed to suckling pups via the colostrum. A technique using a Baermann apparatus may allow recovering hookworm larvae from fresh host's tissues and from pups' stomach contents.

Materials

Scalpel and scalpel blades or sharp scissors; plastic bags; plastic pipette; gauze swabs; plastic conic tube (50ml); modified Baermann apparatus*; stereo (dissecting) microscope (SZ51, Olympus©). **Baermann apparatus:* assemble a plastic cone (top of a 1.5L platic bottle) with a 50ml plastic conic tube. The main difference with a general Baermann system is the absence of tape to collect the bottom content of the tube.

Method

- Collect about 100 to 200 grams of fresh tissues into a plastic bag or about 10ml of milk from the pup's stomach (see Appendix 5.2). Tissues include blubber (abdominal, axillary and inguinal), mammary glands and even muscles, stomach content of newborns, placentas.
- Cut tissues into very small pieces using a scalpel or sharp scissors. Be careful: do not contaminate tissue with sand or tap water (both may contain other nematode larvae).
- Place the pieces of tissues or pour the milk volume onto the gauze, on the top of the funnel (plastic cone of the Baermann apparatus).
- Pour warm water (25-30ºC) until the tissue pieces are submerged or top up to 1 cm below the edge of the funnel.
- Leave the system for 4-6 hours at ambient temperature.
- Collect the bottom 20 mL of the tube from the Baermann apparatus in another conic tube using a pipette and examine under stereomicroscope (from x10 to x35 magnification).

Appendix 5.2
SOP for sampling stomach contents of NZSL pups

Principle

To investigate transmammary transmission of hookworm larvae in NZSLs, pups' stomach contents are examined for infective larvae under a microscope.

Materials

2 solid straps (30 to 50 cm long); a 25ml syringe; a plastic rubber tubing (diameter between 1 and 1.5cm, 50cm-lomg) with a 2 cm-notch at 2 cm from the "oesophageal" extremity to facilitate the passage of milk in the tube; lubricant gel; a plastic container with a screw top.

Method

- Immobilize the pup using manual restraint techniques and keep the pup's mouth opened with using the two straps (one for each jaw).
- Introduce the tubing (with lubricant at the extremity) in the oesophagus (be careful: do not introduce the tube into the trachea).
- Aspirate about 10ml of stomach content using the syringe connected to the tubing.
- Disconnect the syringe from the tubing and purge the syringe content into the plastic container.
- Remove slowly the tubing from the oesophagus; avoid any discharge of liquid from the tube (liquid could be swallowed into the trachea) by putting the thumb at the extremity.
- Rinse the tube with water and allow drying before the next use.
- Examine the content under a stereomicroscope after processing it through a Baermann apparatus (*see* Appendix 5.1).

APPENDIX 5.3

SOP for collecting adult hookworms from the small intestines of NZSL pups

Principle

To assess hookworm burden in NZSL pups, the intestinal content is collected at necropsy for every pup.

Materials

Sharp scissors; a bucket and running water; sieves (openings 75 and 150µm); jars; 10% formalin.

Method

- Cut away all the mesentery around the intestines so they are relatively straight
separate the large and small intestine
- Get some really good small sharp scissors and run them along the intestine, opening up the lumen
- Rinse the lumen (and rub with fingers) under softly flowing water into a bucket
- Empty bucket into a fine grain sieve (openings 150µm)
- Empty contents of sieve into jar with formalin.

APPENDIX 5.4

SOP for worm counting in NZSL pups

Principle

Intestinal contents collected at necropsy of all NZSL pups infected with hookworms are sieved and hookworms are counted to assess the burden.

Materials

Glass jar (250ml); stick; “ladle” (capacity of 25ml); sieve (opening 150 µm); manual counter.

Method

- Transfer the specimens from the jar filled with 10% formalin (see Appendix 5.3) into a 250mL glass jar and fill it with water to 250mL.
- Mix thoroughly with a stick and immediately sample 25mL from the jar with using a 25ml “ladle”.
- Pour these 25ml onto a sieve (opening 150 µm).
- Record the number of adult hookworms with a manual counter.
- Multiply the number of worms counted by 10 (dilution factor).

APPENDIX 5.5

SOP for examination of faeces from NZSL pups

Principle

Finding hookworm eggs in pups' faeces contributes to the diagnosis of infection in NZSL pups. The faecal egg count indicates the likely parasitic burden. Strongylid egg will float in a medium with a specific gravity of 1.2 as the saturated NaCl.

Materials

Plastic bags or plastic vials; plastic gloves; plastic loops; saturated NaCl solution (prepared with tablets dissolved into water in the field); a little glass "universal" bottle (volume 28ml); a small tea sieve (aperture 150μm); a spoon; a 50ml-bowl; a plastic pipette; microscopic slides and coverslips; a McMaster slide (Advanced Equine Products, ISSAQUAH, WA, USA); a light microscope (Olympus©).

Method

- **Direct smear**: roll a swab of faeces on a drop of NaCl on a slide and place a coverslip on top of it. Examine for hookworm eggs under the light microscope (low magnification first: x40, then x100 and x200).

- **Faecal flotation**: break 1 gram of faeces (a coffee spoon) into saturated saline water. Fill the universal bottle with the mix and top up with saline water until a meniscus is formed at the top. Place a cover slip and allow the eggs to concentrate underneath the coverslip for 10 minutes. Place the coverslip onto a slide and examine under the light microscope.

- **Modified McMaster method** for egg count: break 2 grams of faeces (a coffee spoon) with saturated NaCl (28ml of liquid=equivalent to one universal bottle) for a total displacement of 30 ml. Work suspension through a coarse sieve (aperture 150μm). Discard retentate. Stir, filtrate and fill the two chambers of the McMaster slide with a pipette. Leave for 5 minutes. The area under each grid is 0.15 ml. Count the number of eggs for both and multiply the result by 50 to obtain the number of eggs/gram.

APPENDIX 6.1

Hookworm burden and faecal egg counts in live (n=14) and necropsied (n=10) NZSL pups for the 2003/2004 and 2004/2005 breeding seasons

Pup no.	Date	Burden	Egg count (epg)	Post-mortem diagnoses*
1	5/2/2004	660	2,700	Arthritis (1); Hookworm infection (2)
2	5/2/2004	770	1,000	Trauma (1); Hookworm infection (2)
3	14/1/2005	1,490	900	Hookworm infection (1); Arthritis (2)
4	27/1/2004	2,310	11,200	Hookworm infection (1)
5	10/2/2004	2,670	7,300	Hookworm infection (1); Septicaemia (2)
6	5/2/2004	3,910	3,400	Hookworm infection (1); Trauma (2)
7	21/1/2004	**	1,000	Trauma (1); Hookworm infection (2)
8	2/2/2004	**	5,600	Hookworm (1); Polyarthritis (2)
9	14/2/2004	**	3,500	Trauma (1); Hookworm infection (2)
10	14/2/2004	**	8,300	Hookworm infection (1)
11	14/1/2005	**	2,000	n/a
12	18/1/2005	**	29,000	n/a
13	25/1/2005	**	40,000+ (30 days-old)	n/a
14	30/1/2005	**	12,000	n/a
15	4/2/2005	**	6,000	n/a
16	4/2/2005	**	8,000	n/a
17	19/1/2004	**	18,000	n/a
18	22/1/2004	**	9,000	n/a
19	21/1/2004	**	3,000	n/a
20	29/1/2004	**	1,000	n/a
21	29/1/2004	**	3,000	n/a
22	29/1/2004	**	4,400 (43 days-old)	n/a
23	5/2/2004	**	9,000 (50 days-old)	n/a
24	5/2/2004	**	200	n/a

*when applicable; ** not known; Pups no. 1 to 10 are necropsied pups and no.11 to 24 are live pups.

APPENDIX 6.2

SOP for blood sampling of

NZSL pups between birth and 2 months of age

Principle

Blood samples allow measuring haematological parameters and investigating the influence of hookworm infection on these data.

Materials

5mL syringes; needles 18G; 10ml-blood tubes containing EDTA (Vacutainer© Becton, Dickinson and Co, NJ, USA).

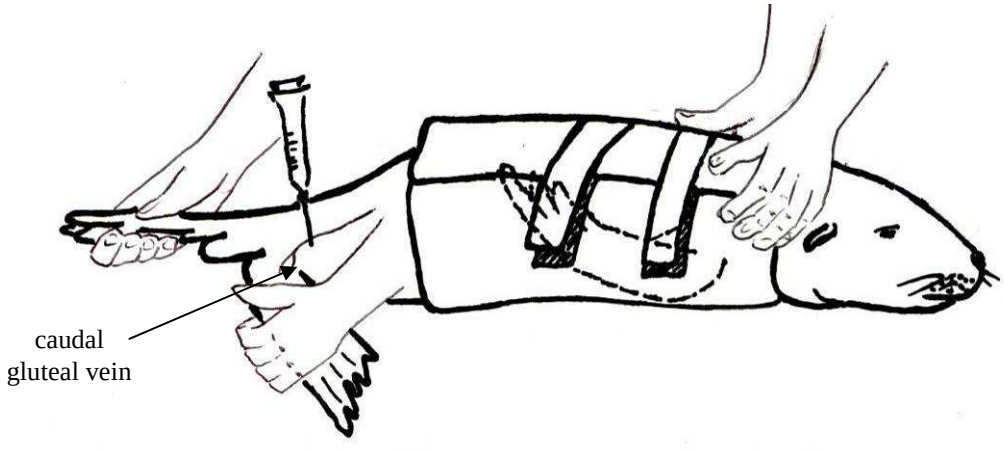
Method

- ***Handling*** (cf figure below). The pup is restrained in a system consisting of a fleece square secured with Velcro© straps. Two persons are necessary to hold the pup and to keep the puncture site immobile.

- ***Blood collection.*** To access the caudal gluteal vein, palpate the two femoral trochanters and draw a virtual triangle with the base of the tail. The vein is parallel to the sacral vertebrae in the area. Puncture the vein with needle mounted on the syringe and withdraw about 2ml of blood.

- ***Blood transfer.*** Blood is immediately transferred into untreated sterile or EDTA tubes (Vacutainer®, USA).

- Carefully detach needle from syringe and place used needle in sharp container.



APPENDIX 6.3

Haematocrit of NZSL pups (1):

2003/2004 breeding season

Pup id.	Sex	Treatment *	Age (days)	Hematocrit (%)
O42	M	0	32	37.0
O43	F	0	36	20.0
O41	M	0	37	37.0
O38	F	0	38	31.0
O39	M	0	38	30.0
O31	F	0	41	21.5
O34	M	0	41	30.0
O23	F	0	42	26.0
O36	M	0	42	26.0
O26	M	0	43	27.0
O29	F	0	44	32.0
O18	F	0	46	28.0
O17	F	0	47	33.0
O20	M	0	47	30.0
O9	M	0	49	38.5
O10	F	0	49	26.0
O5	M	0	52	26.5
O3	F	0	53	27.0

Pup id.	Sex	Treatment *	Age (days)	Hematocrit (%)
Y21	M	1	40	27.0
Y20	M	1	41	28.0
Y17	F	1	44	25.0
Y12	M	1	45	24.0
Y14	M	1	45	33.0
Y13	F	1	45	28.5
Y10	F	1	46	27.0
Y7	M	1	47	31.0
Y8	F	1	47	36.0
Y9	M	1	47	32.0
Y5	M	1	49	29.0
Y3	F	1	51	32.0
Y2	M	1	52	29.0

* Treatment 0=infected pups; 1=pups treated with ivermectin (200 µg/kg; Ivomec® injection for cattle, sheep and pig, 10g/L ivermectin, Merial New Zealand Ltd).

Pups were injected three times (at 2 days, 7 days and 1 month of age).

APPENDIX 6.3 (cont.)

Haematocrit of NZSL pups (2):

2004/2005 breeding season

Pup id.	Sex	Treatment *	Age (days)	Hematocrit (%)
O2	M	0	5	49.0
O2	M	0	18	37.5
O2	M	0	25	35.5
O2	M	0	50	32.0
O3	F	0	15	36.0
O3	F	0	53	27.0
O4	M	0	8	39.5
O4	M	0	23	33.5
O4	M	0	55	26.0
O8	F	0	53	34.5
O14	F	0	1	43.5
O14	F	0	14	43.0
O15	M	0	2	46.0
O15	M	0	8	30.0
O15	M	0	14	30.5
O15	M	0	51	28.8
O16	F	0	1	49.0
O16	F	0	7	47.0
O16	F	0	13	36.5
O18	M	0	7	43.0
O18	M	0	14	34.5
O18	M	0	47	30.0
O20	M	0	9	46.0
O20	M	0	44	32.5
O24	F	0	2	59.5
O24	F	0	38	34.5

Pup id.	Sex	Treatment *	Age (days)	Hematocrit (%)
Y1	F	1	5	43.0
Y1	F	1	25	42.5
Y1	F	1	58	47.5
Y4	M	1	1	53.5
Y4	M	1	8	50.0
Y4	M	1	55	26.0
Y8	F	1	1	51.0
Y8	F	1	21	39.0
Y14	F	1	1	49.0
Y15	M	1	1	43.0
Y15	M	1	11	46.5
Y16	F	1	47	35.0
Y18	M	1	1	45.0
Y18	M	1	7	38.0
Y18	M	1	14	43.5
Y18	M	1	46	35.5
Y20	M	1	44	38.0

* Treatment 0=infected pups; 1=pups treated with ivermectin (200 µg/kg; Ivomec® injection for cattle, sheep and pig, 10g/L ivermectin, Merial New Zealand Ltd). Pups were injected three times (at 2 days, 7 days and 1 month of age).

APPENDIX 6.4

Unopette© procedure for WBC and platelet counts

Principle

Analysis conducted with the Unopette© system (Becton, Dickinson and Co, NJ, USA) allows obtaining WBC and platelet counts.

Materials

Plastic container with screw cap, containing 0.475ml of lysis solution; solution: acetic acid 3%; capillary pipette 25µl.

Method

- Puncture the diaphragm in the neck of the reservoir with the tip of the capillary pipette. Fill in the capillary pipette with blood. When blood reaches the end of the capillary bore in the neck of the pipette, filling stops automatically. Wipe any blood off the outside of the tube.
- With one hand, gently squeeze the reservoir to force some air out and maintain pressure on the reservoir. With the other hand, cover the upper opening of the capillary overflow chamber with the index finger and seat the capillary pipette holder in the reservoir neck.
- Release pressure on the reservoir and remove your finger from the overflow chamber opening. Suction will draw the blood into the diluent in the reservoir.
- Squeeze the reservoir gently two or three times to rinse the capillary tube without letting the diluent out.
- Remove the pipette from the reservoir. Invert and fill the capillary pipette by gentle pressure on the reservoir. Load the counting chamber of the haematocytometer (Glasstic©) by gently squeezing the reservoir while touching the tip of the pipette against the edge of the coverglass on the counting chamber.
- Place the loaded haematocytometer on the microscope. Allow 10 minutes for the cells to settle.
- Count the WBCs in all 9 fields, each one divided into 9 smaller squares. Count of all the cells within each square, including cells touching the lines inside the square. **Do not count any cells that touch the lines outside a square.** Multiply the count by 100 to obtain the number of WBCs per µl.

APPENDIX 6.5

SOP for preparation of blood smears and May-Grünwald Giemsa stain

Preparation of blood smears:

- apply a drop of blood on a clean , dry and dust-free glass microscope slide
- spread the blood over the slide using the smooth end of another glass microscope slide as spreader. There should be a 45º angle between the smooth edge of the spreader and the smear slide. Spreading has to be done in a single, rapid move to ensure a homogenous smear
- allow the blood smear to dry at ambient air.

Staining and dehydrating solutions

May-Grunwald: stock 0.3% May-Grünwald solution, diluted 1:1 with phosphate buffer (pH= 7.0); ***Giemsa:*** stock Giemsa solution, diluted 1:9 in phosphate buffer (pH=7.0); ***50/50 buffer/acetone***; ***xylene.***

May-Grünwald Giemsa (MGG) method:

- Air dry slides
- Fix in 100% ethanol for 2-3 minutes at room temperature
- Stain for 15 minutes in May-Grunwald stain freshly diluted with an equal volume of buffered distilled water, pH=7.0
- Stain for 10 minutes in Giemsa stain freshly diluted with buffered distilled water, pH=7.0
- Wash in running tap-water and then leave for 3-4 minutes in buffered distilled water, pH=7.0
- Rinse in 50/50 buffer/acetone
- Dehydrate in acetone
- Clear in xylene
- Allow to dry and mount with a coverslip, using DPX neutral mounting resin.

Luna LG.1993. Histopathologic methods and color atlas of special stains and tissue artifacts. American Histolabs Publication Department, Gaithersburg, MD, 767pp.

APPENDIX 6.6

WBC differential counts in NZSL pups (2003/2004)

Pup id.	Sex	Age (days)	Lactating female id. (age-yrs)	Band Neutrophils	Segmented Neutrophils	Basophils	Eosinophils	Lymphocytes	Small Lymphocytes	Large lymphocytes	Monocytes
O42	M	32	.	0	77	0	7	4	.	.	12
O43	F	36	1472 (13)	0	72	0	0	20	13	7	8
Y15	F	36	1437 (11)	1	72	0	0	21	16	5	6
O16	F	36	1382 (unkn.)	0	74	0	0	22	13	9	4
O41	M	37	1412 (unkn.)	0	70	0	2	20	17	3	8
O38	F	38	(unkn.)	0	64	0	2	33	.	.	1
Y8	M	39	1493 (unkn.)	1	58	0	0	36	31	5	5
Y7	F	39	1498 (unkn.)	0	76	0	0	22	16	6	2
O39	M	39	1388 (unkn.)	0	65	0	3	24	19	5	8
Y2	M	41	1438 (11)	1	69	0	1	21	13	8	8
O31	F	41	1461 (12)	0	42	0	1	55	48	7	2
O34	M	41	1470 (unkn.)	1	74	0	3	19	10	9	4
O36	M	42	1404 (unkn.)	0	55	0	6	34	.	.	5
O26	M	43	1419 (13)	1	63	0	1	29	15	14	6
O29	F	44	1485 (14)	3	49	0	4	40	31	9	4
O23	F	44	1433 (14)	0	73	0	1	22	20	2	4
Y21	M	44	1462 (15)	0	63	0	0	36	27	9	1
O18	F	46	1456 (11)	0	52	0	3	40	30	10	5
O 17	F	47	1398 (12)	0	57	0	14	25	.	.	4
O20	M	47	1484 (unkn.)	0	60	0	8	27	20	7	5
Y14	M	47	1424 (13)	0	64	0	0	34	16	18	2
Y20	M	47	(unkn.)	0	72	0	0	20	13	7	8
Y13	F	48	1499 (17)	0	73	0	0	23	15	8	4
Y17	F	48	1398 (12)	0	50	0	0	47	21	26	3
Y12	M	48	1381 (14)	0	48	0	4	47	37	10	1
Y10	F	48	1439 (10)	0	57	0	2	36	20	16	5
O9	M	49	1400 (11)	0	59	0	9	24	.	.	8
O10	F	49	(unkn.)	0	50	0	0	47	21	26	3
Y9	M	49	(unkn.)	0	74	0	2	16	9	7	8
Y8	M	50	1493 (unkn.)	1	69	0	0	28	16	12	2
Y7	F	50	1498 (11)	0	72	0	2	19	11	8	7
O5	F	51	1492 (12)	1	68	0	4	25	17	8	2
O5	M	52	1492 (12)	0	51	0	16	29	.	.	4
Y5	F	52	1492 (12)	0	69	0	1	28	16	12	2
Y3	F	52	1385 (12)	0	69	0	1	23	13	10	7
O3	F	53	1385 (12)	0	64	0	10	22	.	.	4
O2	M	53	1438 (11)	0	64	0	11	22	.	.	3
Y2	M	53	1438 (11)	1	63	0	1	29	15	14	6

APPENDIX 6.5 (cont.)

WBC differential counts in NZSL pups (2004/2005)

Pup id.	Sex	Age (days)	Lactating female id. (age-yrs)	Band Neutrophils	Segmented Neutrophils	Basophils	Eosinophils	Lymphocytes	Small Lymphocytes	Large lymphocytes	Monocytes
Y1	F	25	1499(18)	0	83	0	0	20	14	6	7
Y1	F	58	1499(18)	0	32	0	0	59	36	23	9
O2	M	5	1473(13)	0	62	0	2	14	7	7	22
O2	M	18	1473(13)	0	60	0	0	24	14	10	16
O2	M	25	1473(13)	0	62	0	0	30	13	17	7
O2	M	50	1473(13)	0	50	0	1	43	32	11	6
O3	F	15	1456(14)	2	63	0	0	22	12	10	11
O3	F	53	1456(14)	1	56	0	0	35	16	19	8
O4	M	8	1369 (unkn.)	0	60	0	4	24	10	14	12
O4	M	23	1369 (unkn.)	0	56	0	0	29	13	16	15
O4	M	55	1369 (unkn.)	0	56	0	0	34	26	8	10
Y4	M	1	1399 (unkn.)	0	38	0	0	27	2	25	35
Y4	M	8	1399 (unkn.)	0	53	0	1	32	10	22	14
Y4	M	20	1399 (unkn.)	2	30	0	0	66	13	53	2
Y4	M	55	1399 (unkn.)	0	69	0	0	29	15	14	2
Y8	F	1	119 (5)	0	84	0	0	5	1	4	11
Y8	F	21	119 (5)	0	68	0	0	17	7	10	15
O8	F	53	87(5)	2	70	0	0	20	10	10	8
O12	F	1	1411 (11)	0	65	0	0	13	6	8	8
O12	F	20	B0448 (6)	2	55	0	0	29	13	16	14
O13	F	2	B0448 (6)	0	52	0	2	6	1	6	40
Y13	F	2	B0179 (6)	0	59	0	1	16	8	8	24
O14	F	1	1441 (12)	0	73	0	3	12	3	9	12
O14	F	14	1441 (12)	0	65	0	1	18	10	8	16
Y14	F	1	1419 (14)	0	64	0	0	16	4	12	20
Y14	F	13	1419 (14)	0	65	0	0	19	9	10	16
O15	M	2	1488 (13)	0	47	0	6	22	4	18	25
O15	M	14	1488 (13)	0	52	0	0	33	20	13	15
O15	M	51	1488 (13)	0	57	0	0	34	19	15	9
Y15	M	1	1453 (unkn.)	0	48	0	0	24	14	10	25
Y15	M	11	1453 (unkn.)	0	58	0	0	21	7	14	21
Y15	M	19	1453 (unkn.)	0	63	0	0	33	12	21	5
O16	F	1	1404 (unkn.)	0	71	0	0	13	7	6	16
O16	F	7	1404 (unkn.)	0	65	0	0	27	22	5	8
O16	F	13	1404 (unkn.)	0	58	0	1	27	13	14	14
Y16	F	47	1364 (14)	3	55	0	0	32	19	13	10
Y18	M	1	1452 (14)	0	49	0	3	28	10	18	23

APPENDIX 7. 1

Biochemical reactions for the most common Enterobacteriaceae

		Enterobacter spp.	*Escherichia coli*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Salmonella* subspp.
Indole production		-	+	-	+	+
Methyl red		-	+	(-)	+	+
Voges-Proskauer (VP)		+	-	+	-	-
Citrate		+	-	+	(-)	+
Urease		-/d	-	+	+	-
Phenylalanine deaminase		-	-	-	+	-
Hydrogen sulphide		-	-	-	+	+
Lysine decarboxylase		+/-	(+)	+	-	+
Ornithine decarboxylase		+	D	-	-	+
Motility (36ºC)		+	(+)	-	+	+
Gelatin liquefaction		-	-	-	+	-
Growth in KCN broth		+	-	+	+	-
ONPG (beta-galactosidase)		+	+	+	-	+/-
Acid from:	Dulcitol	(-)	D	d	-	+/-
	Inositol	+/-	-	+	-	+
	Lactose	+	+	+	-	-
	Maltose	+	+	+	+	+
	Mannitol	+	+	+	-	+
	Mannose	+	+	+	-	+
	Rhamnose	+	(+)	+	-	+
	Sorbitol	+	+	+	-	+
	Sucrose	+	D	+	+	-
	Xylose	+	+	+	+	+
Mucoid colonies		+/-	(-)	+	-	-

+ : 90-100% strains positive / **(+):** 76-89% positive / **d:** 26-89% positive / **(-):** 11-25% positive / **-** : 0-10% positive.

From: Quinn PJ, Carter ME, Markey BK, Carter GR (1994) Clinical Veterinary Microbiology. Ed. Wolfe (England), 648 pp.

APPENDIX 7. 2

Biochemical properties and composition of MacConkey and XLD agars

MacConkey agar

Fermentable sugar: lactose.

pH indicator: neutral red (pale straw at pH 8 and pink at pH 6.8).

Inhibitors: bile salts and crystal violet (anti-Gram-positive bacteria).

Reactions: if the bacterium can ferment the lactose, acidic metabolic products are produced and the medium and colonies are pink (lactose-fermenter). If the organism is unable to utilise the lactose, then it attacks the peptone (nitrogen source) in the medium with resulting alkaline metabolic products and the medium and colonies are pale straw-coloured (non-lactose-fermenter).

XLD (xylose-lysine-deoxycholate) medium

Fermentable sugars: lactose, sucrose and xylose.

pH indicator: phenol red (red at pH 8.2 and yellow at pH 6.4).

Other substrates: lysine and chemicals for detecting hydrogen sulphide (H_2S) production.

Inhibitor: bile salts (sodium deoxycholate).

Reactions: salmonellae will first ferment the xylose creating a temporary acid reaction but this is reversed by the subsequent decarboxylation of lysine with alkaline metabolic products. Superimposed on the red (alkaline) colonies is the production of hydrogen sulphide, so most salmonellae have red colonies with a black centre. *Edwardsiella tarda* also gives this reaction although the H_2S production is less marked and the periphery of the colonies tends to be a yellowish-red colour. The large amount of acid produced by enterobacteria that can ferment either lactose or sucrose, or both, prevents the reversion to alkaline conditions even if the bacterium is able to decarboxylate the lysine.

In: Quinn PJ, Carter ME, Markey BK, Carter GR (1994) Clinical Veterinary Microbiology. Ed. Wolfe (England), 648 pp.

Fig. 1.*Klebsiella pneumoniae* on MacConkey agar is characterised by pink or cream mucoid colonies.

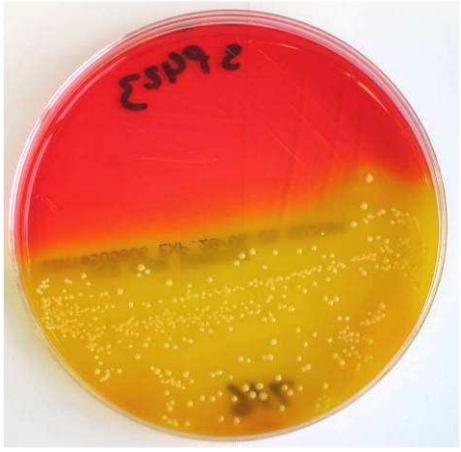


Fig. 2. *Escherichia coli* on XLD agar shows typical small white colonies with a yellow (acid) background.

APPENDIX 7. 3

Antimicrobial sensitivity testing for *Klebsiella pneumoniae* isolates from NZSL pups.

SOP for disk diffusion method on Mueller-Hinton agar.

Principle

Bacterial isolates are grown on a plate in the presence of disks containing antimicrobial agents. The zone of inhibition of bacterial growth around the disk indicates the level of sensitivity of the bacteria to this particular antimicrobial agent.

Materials

2% Blood Agar plates; saline solution; Mueller-Hinton Agar plates (Global Science, Pierce, Rockford, IL); Antibiotic disks (BioRad, Hercules, CA).

Methods

- Culture frozen samples of isolates on 2% Blood Agar plates
- After a 24 hour-incubation period, dissolve one colony from each sample in normal saline solution and apply on three layers on Mueller-Hinton agar
- Simultaneously apply antibiotic disks on the agar for each isolate and incubate plates for 24 hours
- Use concordance charts between diameters of inhibition and susceptibility breakpoints provided by the manufacturer (BioRad, Hercules, CA) to interpret diameters of inhibited zones.

Antimicrobial agents tested with the disk diffusion method: Enrofloxacin (5 μg): ENR 5 / Cefalexin (30 μg): CL 30; Cefotaxime (5 μg): CTX 5; Ampicillin (10 μg): AMP 10; Gentamicin (10 μg): CN 10; Tetracycline (30 μg): TE 30; Penicillin (2 μg): P2; Amoxycillin + Clavulanic Acid (20 μg/10 μg): AMC 30; Trimethoprim Sulfamethoxazole (1.25 μg/23.75 μg): SXT 25.

APPENDIX 7. 3 (cont.)

Disk diffusion method on Mueller-Hinton agar:

Results (1)

Origin of isolates:
2000/2001:
SL243: Pup / tissue: liver / phenotype: *Klebsiella oxytoca*
2001/2002:
SL73, 80, 95, 100, 102, 104, 108, 111, 112, 126: Pup / tissue: lymph nodes (cephalic or thoracic region) / phenotype: *Klebsiella pneumoniae*
2002/2003:
SL 147, 150, 171, 177, 180, 183, 191, 201: Pup / tissue: lymph nodes (cephalic or thoracic region); SL 218: Pup/ tissue: swab carpal joint / phenotype: *Klebsiella pneumoniae.*

Samples	**ENR5**	**CL30**	**SXT25**	**CTX5**	**AMP10**	**CN10**	**TE30**	**AMC30**	**P2**
SL243	28	21	24	27	9	20	25	23	0
SL73	30	22	27	31	0	19	14	15	0
SL80	30	22	27	31	9	21	16	17	0
SL95	28	21	24	27	0	20	12	13	0
SL100	30	21	26	29	0	19	8	12	0
SL102	29	22	25	27	0	19	13	14	0
SL104	28	22	27	30	16	20	14	16	0
SL108	24	22	27	29	0	18	14	16	0
SL111	29	22	29	32	10	20	13	16	0
SL112	29	23	27	31	11	21	15	17	0
SL126	22	20	24	26	9	20	14	16	0
SL106	27	19	24	26	0	16	12	22	0
SL147	26	20	23	25	0	18	13	14	0
SL150	27	22	25	27	9	20	15	24	0
SL171	26	20	26	27	0	18	11	23	0
SL177	26	20	24	25	0	19	13	14	0
SL180	29	20	27	28	0	18	12	21	0
SL183	27	21	23	26	0	18	13	24	0
SL191	26	20	23	27	0	19	13	23	0
SL201	27	22	23	26	0	19	12	23	0
SL218	28	20	25	28	0	19	14	23	0

APPENDIX 7. 3 (cont.)

Disk diffusion method on Mueller-Hinton agar:

Results (2)

Concordance charts:

Antimicrobial	ENR5	CL30	SXT25	CTX5	AMP10	CN10	TE30	AMC30	P2
Susceptible	≥ 23	≥18	≥16	≥18	≥17	≥15	≥19	≥18	≥17 *
Intermediate	17-22	15-17	11-15	15-17	14-16	13-14	15-18	14-17	14-16*
Resistant	≤16	≤14	≤10	≤14	≤13	≤12	≤14	≤13	≤13*

*human reference values

These ranges of reference for antimicrobial susceptibility are valid for Enterobacteriacae isolated in dogs. Disk diffusion testing was not used here to determine the antimicrobial susceptibility but to investigate the similarities between isolates. *K.oxytoca* and *K.pneumoniae* isolates of humans and adult NZSL were tested with the same method for comparison.

Years	***phenotype***	ENR5	CL30	SXT25	CTX5	AMP10	CN10	TE30	AMC30	P2
2000/ 2001	*K.oxytoca* (1)	S*	S	S	S	R	S	S	S	R
2001/ 2002	*K.pneumoniae* (12)	11 S, I	12 S	12 S	12 S	I, 11 R	12 S	I, 11 R	2 S, 7 I, 3 R	12 R
2002/ 2003	*K.pneumoniae* (10)	10 S	10 S	10 S	10 S	10 R	10 S	2 I, 8 R	8 S, 2 I	10 R
2003	*K.pneumoniae* (1) Adult male	S	S	S	S	I	S	S	I	R
2003	*K.oxytoca* (1) Human	S	S	S	S	R	S	R	S	R
2004	*K.pneumoniae* (4) Human	4 S	4 S	4 S	4 S	4 R	4 S	3 S, R	4 S	4 R

*Susceptible (S), Intermediate (I), Resistant (R)

APPENDIX 7. 4

Antimicrobial sensitivity testing for *Klebsiella pneumoniae* isolates from NZSL pups.

SOP for determination of Minimal Inhibitory Concentration (MIC)

This protocol is largely as described in Martin Situmbeko's Masterate thesis (IVABS, Massey University, 2004) entitled "MIC of selected antimicrobial agents against mastitis causing Staphylococcus aureus isolates from dairy cows in New Zealand in 2002".

Principle

MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a micro-organism after overnight incubation.

Materials and Reagents

- Disposable micro-dilution tray and disposable inoculum reservoirs (Nunc Microwell Nunclon®); electric pipettor and pipette tips (Pipetman Ultra© Gibson, France); multi-channelled inoculator.
- Mueller Hinton broth (Mueller-Hinton broth with 2% sodium chloride for oxacillin and Cation-adjusted Mueller-Hinton broth for tetracycline testing); stock solution.

Method

1.Preparation of micro-dilution trays with an antimicrobial

- Take an antimicrobial stock solution vial from the -70°C freezer and place on bench to thaw to room temperature.
- Unpack sterile disposable micro-dilution tray and disposable inoculum reservoir trays. Label the inoculum reservoirs (columns) "A" to "H".
- Using electric pipettor, dispense the sterile Mueller-Hinton broth onto inoculum reservoir tray "A" so that the total amount is 20 ml after addition of stock solution.
- Using the electric pipettor, add the complementary volume of aliquot of thawed stock solution to inoculum reservoir tray "A". Discard the tip into disinfectant.
- Use the electric pipettor fixed with a sterile disposable pipette tip, mix solution well by withdrawing and releasing the solution 5 to 6 times.
- Transfer 10 ml of solution from tray "A" to tray "B" using the electric pipettor. Discard the pipette tip, load a new disposable pipette tip and mix thoroughly.

- Transfer 10 ml of solution from tray “B” to tray “C”. Discard the pipette, fix a new tip, mix thoroughly and continue the dilutions by repeating the procedure up to tray “H”, being certain to change pipettes between trays to prevent carryover of antibiotic on the external surface of the pipette.
- Using adjustable multi-channelled inoculator (G.G. Midwinter, IVABS, Palmerston North) loaded with sterile disposable tips, transfer 0.05 ml (50μg) of solution of tray “A”, in wells from row “A1” to row “A11”. Row “A12” is filled with 0.5 ml of Mueller-Hinton broth (Mueller-Hinton broth with 2% NaCl for oxacillin and CAMHB for tetracycline) without antimicrobial agent.

2. Bacterial inoculum preparation by direct colony suspension method

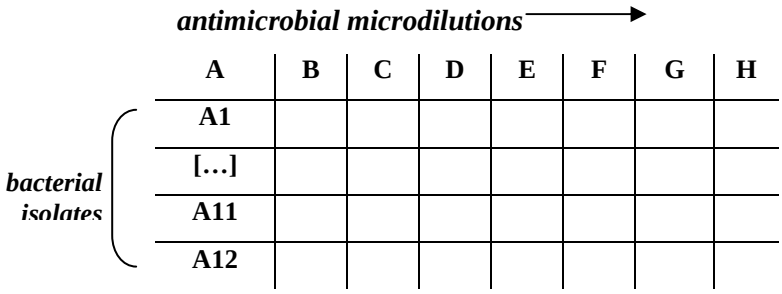
- Using a sterile cotton swab, pick 4 to 5 isolated colonies from an overnight nutrient agar culture and transfer to a tube containing 5 ml of sterile distilled water or 0.9% saline. Mix well by vortex.
- Directly adjust turbidity visually by adding either sterile distilled water (or 0.9% saline) or more colonies as required to equivalent that of a 0.5 McFarland standard. This is achieved using good light by visually comparing the appearance of black lines through the inoculum and McFarland standard suspensions. (**Note:** Turbidity adjusted to this standard contains approximately 1 - 2 x 10^8 CFU/ml.)
- Dispense 9.9 mls of sterile Mueller-Hinton broth (cation-adjusted Mueller-Hinton broth for tetracycline or Mueller-Hinton broth with 2% sodium chloride for oxacillin testing) to a sterile inoculum reservoir tray using a broth dispenser.
- Add 0.1 ml of the adjusted inoculum using the adjustable micro-pipettor fixed with a sterile tip. Mix well by gently tilting the inoculum reservoir dish five to six times to and fro. (**Note:** This is 1:100 dilution ratio and this type of inoculum contains approximately 1-2 x 10^6 CFU/ml.)

3. Bacterial inoculation in the micro-dilution trays

- Using the adjustable multi-channelled inoculator, withdraw 0.05 ml (50μl) volume of inoculum suspension in the reservoir dish. Check that the volume in the tips is correct, uniform and without air bubbles.
- Carefully dispense the inoculum into the antimicrobial solution in column 1 of the micro-dilution tray. Mix the inoculum and the antimicrobial solution well by sucking up and down 4-5 times before discarding the tips in disinfectant. (**Note:** This results to a concentration of approximately 5 x 10^5 CFU/ml or 5 x 10^4 CFU/well.)

- Record the inoculated micro-dilution tray and isolate number on the MIC recording sheet. Repeat for the other isolates from column 2 up to column 11.

- In column 12, add 0.05 ml of plain Mueller-Hinton (cation-adjusted Mueller-Hinton broth for tetracycline and Mueller-Hinton with 2% sodium chloride for oxacillin testing) and is recorded as sterility control column.



4. Quality Control Scheme for broth micro-dilution MIC determination

Quality control strains

- Use a quality control strain: *Staphylococcus aureus* ATCC 29213.

- Maintain bacterial isolates at -70°C in glycerol broth with 15% glycerol.

- Culture all isolates on blood agar followed by sub-culture on nutrient agar prior to MIC determination.

- Make sure that quality control strain MIC results are within the NCCLS quality control ranges specified for the respective antimicrobial agent.

- Record MIC results for QC strain on Antimicrobial Susceptibility Quality Control Test Record.

Other quality controls

- Include sterility control wells (wells without inoculum and antimicrobial agent) on each micro-dilution tray as row 12.

- Prepare inoculum count verification plates from inoculum reservoir dish immediately after inoculating the micro-dilution trays.

- Prepare purity control plates from the same inoculum reservoir dish as for the inoculum count verification.

- Prepare sterility control plates from broth in the broth dispenser immediately after the last isolate of each batch.

Required quality control results

- Check that MICs for quality control strain are within the NCCLS quality control range specified for the respective antimicrobial agent.
- Check that sterility control wells are free of any growth.
- Check that inoculum count verification plates have approximately 100 colonies.
- Check that purity control plates show a good and pure growth.
- Check that sterility control plates show no bacterial growth.

5. Verification of a correct inoculum size

- Using the adjustable micro-pipettor fixed with sterile tip, transfer 0.01 ml aliquot from the inoculum reservoir to 10 ml of 0.9% sterile saline contained in metal-capped tube immediately after inoculation.
- Discard the tip in disinfectant and mix well by vortex.
- Fix another sterile tip to the adjustable micro-pipettor and transfer 0.1 ml aliquot onto the surface of blood agar. Discard the tip in disinfectant.
- Using a bent glass rod dipped in alcohol, passed through the Bunsen burner flame and waited to cool, spread the inoculum over the entire surface of the blood agar plate evenly.
- Incubate at 37°C for 24 hrs and count the colonies.

6. Preparation of the purity plate

- The purity plate is prepared from the same isolate as the inoculum count verification plate immediately after inoculation.
- Using the adjustable micro-pipettor fixed with sterile tip, inoculate the blood agar surface with 0.01 ml aliquot from the inoculum reservoir dish.
- Using a sterile non-toxic cotton swab, spread the inoculum on the plate

7. Preparation of the sterility plate

- Dispense a few drops of Mueller-Hinton broth from the broth dispenser onto the surface of the blood agar immediately after the last isolate of the batch.
- Using a sterile non-toxic cotton swab, spread the inoculum on the plate

APPENDIX 7. 5

SOP for testing of the production of Extended Spectrum Beta-Lactamase (ESBL)

Principle

Enterobacteria may acquire some resistance via plasmids. Bacteria involved in nosocomial outbreaks are ESBL producing.

Method

- Follow the NCCLS procedures were followed (NCCLS Volume 22, pg 69 Table 9A: Screening and Confirmatory Tests for ESBLs)
- Use two Quality Control Organisms from the Reference Culture Collection (ESR, Porirua, Wellington, New Zealand): *Escherichia coli* (916, ATCC 25922) - negative control and *Klebsiella pneumoniae* (3681, ATCC 700603) - positive control with production of Beta-lactamases.
- Inoculate the Mueller-Hinton agar plates with a lawn of bacterial suspension.
- Apply the antimicrobial disks within 15min and leave the plates in the incubator overnight (CO_2, at 37ºC).
- Record the diameter of the zone of inhibition.

Results

Quality Controls (QC):

-*E.coli* <=2mm in both antimicrobials and the limit increases with Clavulanic acid

-*K.pneumoniae* >=5mm in the Ceftazidime-Clavulanic acid and >= 3mm in the Cefotaxime- Clavulanic acid.

Sample	Ceftazidime	Ceftazidime/ Clavulanic acid	Cefotaxime	Cefotaxime/ Clavulanic acid
SL71	30mm	30mm	33mm	33mm
SL90	31mm	31mm	34mm	34mm
SL113	30mm	30mm	32mm	32mm
SL151	30mm	30mm	32mm	32mm
SL156	30mm	31mm	32mm	32mm
SL185	30mm	30mm	32mm	32mm
E.coli QC	31mm	32mm	34mm	34mm
K.pneumoniae QC	14mm	24mm	21mm	27mm

Interpretation of results:

A >= 5mm increase in a zone diameter for either antimicrobial between antimicrobials alone and combined with Clavulanic acid meant ESBL production. **Conclusion*:*** none of the samples tested produced ESBLs.

APPENDIX 7. 6

SOP for *XBa*I macrorestriction-PFGE DNA analysis of *Klebsiella pneumoniae* isolates from NZSLs

Principle

PFGE is a molecular typing technique commonly used to determine the relatedness of a group of bacterial isolates by digesting chromosomal DNA with a restricting enzyme.

Reagents

Brain-Heart Infusion (BHI) broth; PETT IV buffer; Low Melt agarose; EDTA solution; Sodium lauroyl sarcosine; NEBuffer (colour-coded 10x NEBuffer for restriction enzyme to provide 100% activity; New England Biolabs Ltd, UK); BSA (Bovine Serum Albumine) buffer; Proteinase K; sterile MQ water (sterile distilled water); TBE (Tris Borate EDTA) buffer (commonly used in all DNA electrophoretic applications to obtain a better resolution of small DNA fragments); restriction buffer; *XBa*I enzyme; Ethidium Bromide solution.

Method

Day 1: **PLUG PREPARATION**

- Grow overnight on Blood Agar (BA) plates and harvest the growth into BHI broth and grow overnight.
- Measure the optical denstity (OD) of the broth at 610nm and take appropriate amt of cells to get an OD of 1.4 in an eppendorf tube.
- Centrifuge at 13000rpm for 5minutes
- Remove supernatant and resuspend cells in 150µl of cold PETT IV buffer.
- Centrifuge for 5 minutes at 13000rpm.
- Remove supernatant and resuspend pellet in 50µl of PETT IV buffer.
- Prepare <u>1% Low Melt Agarose</u> as follows:

> *Add 4ml of PETT IV buffer to 40mg of Low Melt agarose heat in a boiling waterbath until the agarose has dissolved. Allow agarose to cool.*

- Add 100µl of molten agarose to the cell suspension and pipette up and down to mix.
- Dispense 100µl of cells/agarose suspension into plug moulds.

- Cool plugs on ice for an hour.

- Prepare Lysis buffer as follows:

0.5M EDTA …….. 20ml of 0.5M per 20ml buffer
1% Sodium lauroyl sarcosine …….. 200mg per 20ml buffer
0.5mg Proteinase K …….. 10mg per 20ml buffer

- Buffer can be stored at –20ºC.

- Place plugs into 1ml of Lysis buffer in Eppendorf tubes.

- Incubate overnight at 56°C.

Day 2 **PLUG WASHING**

- Transfer the plugs into plastic universals containing 10ml TE buffer each.
- Place universals on ice and incubate for 1 hour on the rocking machine.
- Drain TE buffer off and replace with another 10 ml of TE buffer.
- Place universals on ice and incubate on rocker for 1 hour.
- Repeat wash step another 3 times.
- Store plugs in 1ml TE buffer at 4°C.

Day 3 **RESTRICTION DIGEST**

- Prepare restriction buffer (*Xba*I)

For each plug add the following and mix:
12µl of restriction buffer (10X NEBuffer 2)
10µl 10X BSA
78µl sterile MQ water

- Flame a glass slide which has been dipped in ethanol (90%).

- Carefully remove the plug from the eppendorf tube and place onto the glass slide. Slice off one third of the plug, using a flamed scalpel.

- Place plug slice into 100µl of restriction buffer and equilibrate on ice for 45 minutes.

- Place the remaining two-thirds of the plug into 1ml TE in an eppendorf and store at 4°C until required.

- Prepare cutting buffer as follows:

For each plug add the following and mix:
10µl of Restriction buffer (10X NEBuffer 2)
10µl 10X BSA
30 Units XbaI (1.5µl of 20U/µl)
78.5µl of sterile MQ water

- Remove restriction buffer and replace with 100µl of cutting buffer and equilibrate on ice for 45 minutes.

- Incubate plugs at 37°C for 12-24 hours.

Day 4 **GEL RUNNING**

- Prepare 1% PFC Agarose as follows:

> *Prepare 80ml agarose for standard mold and 140ml for wide/long mold.*
> *e.g. for 80ml mold*
> *0.8g agarose*
> *80ml 0.5X TBE buffer*
> *Heat for around 3 minutes in microwave until agarose is dissolved. Place agarose at about 50℃ to cool for 5 minutes.*

- Prepare gel mold and comb.

- Pour into gel mold, popping any bubbles that come up at the corners.

- Allow gel to solidify for 1 hour and then carefully remove the comb.

- Pre-electrophorese the gel at 6V/Cm, 5-5 sec., for 1.5 hours, Angle 120oC

- Remove the gel from the chamber and pipette the remaining buffer out of the wells.

- Tip plugs out of eppendorf tubes onto flamed glass slides, then slide plugs into wells. Push plugs to the bottom front of the well using a 'hockey stick' (load marker plugs also).

- Fill wells with molten 1% agarose and allow setting.

- Place gel in electrophoresis chamber and run at the following conditions:

6V/Cm
Angle 120oC
20 hour
Initial switch= 5 second
Final switch= 55 seconds

Day 5 **STAINING AND PHOTOGRAPHING GEL**

CAUTION ! **Ethidium bromide (EB)** is carcinogenic

WEAR GLOVES !

- Remove gel from chamber and slip it off the black plate into EB solution (1 µg/ml).

- Stain gel for 10 minutes.

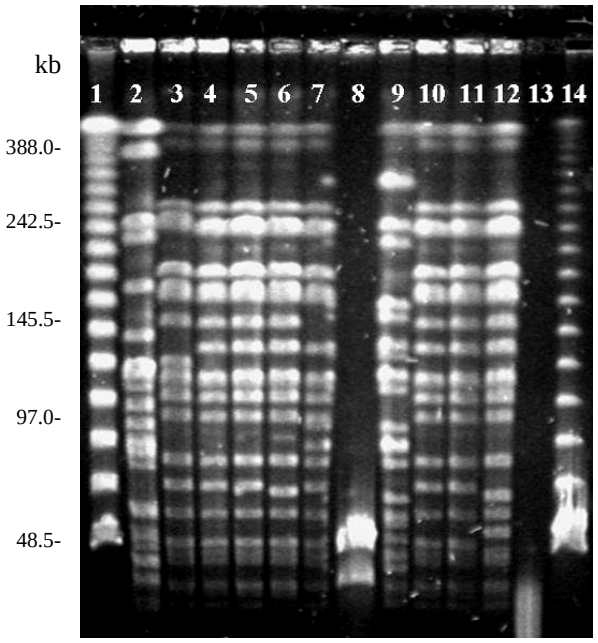
- Transfer gel to a plastic container containing MQ water and rinse briefly.

- Photograph gel on GelDoc© 2000.

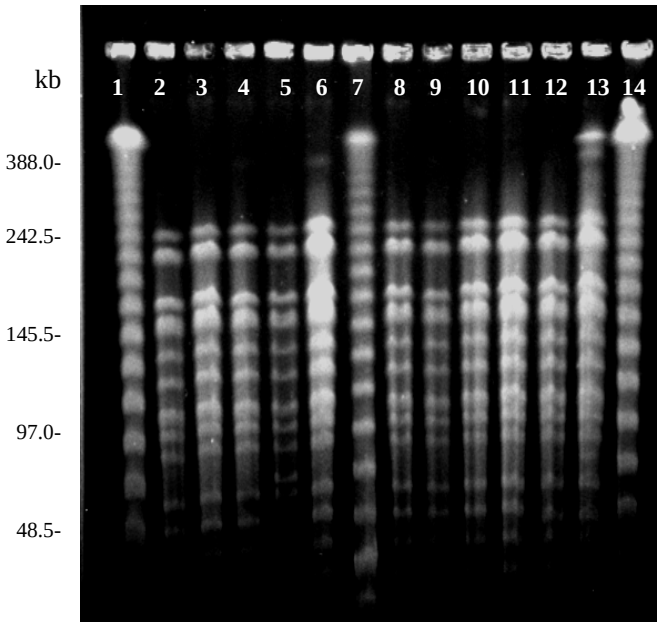
APPENDIX 7. 6 (cont.)

PFGE analysis of *Klebsiella pneumoniae*

isolates from NZSLs: Results



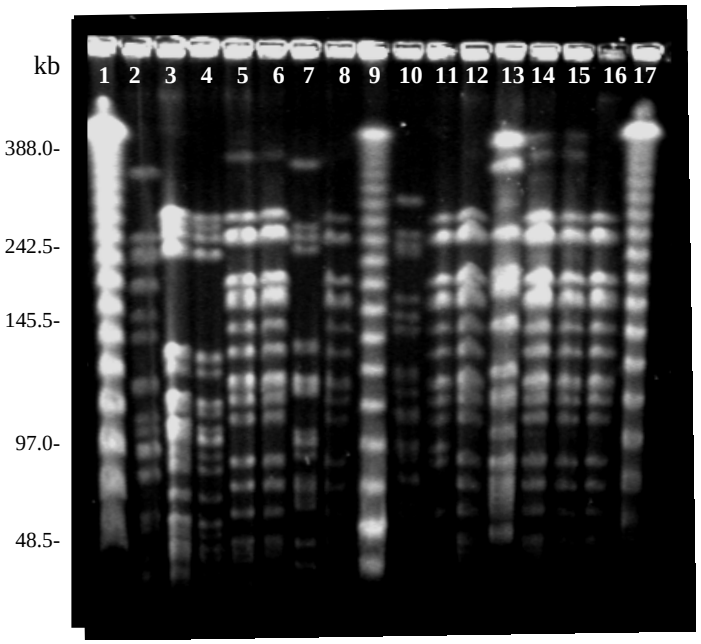
lane no.	sample
1	lambda ladder
2	*Kpn* human isolate 1
3	*Kpn* pup 2002/2003
4	*Kpn* pup 2001/2002
5	*Kpn* pup 2002/2003
6	*Kpn* pup 2002/2003
7	*Kpn* pup 2001/2002
8	faulty plug
9	*Kpn* human isolate 2
10	*Kpn* pup 2002/2003
11	*Kpn* pup 2002/2003
12	*Kpn* pup 2001/2002
13	*Kpn* human isolate 3
14	low range ladder



A, B: isolates from the same pup

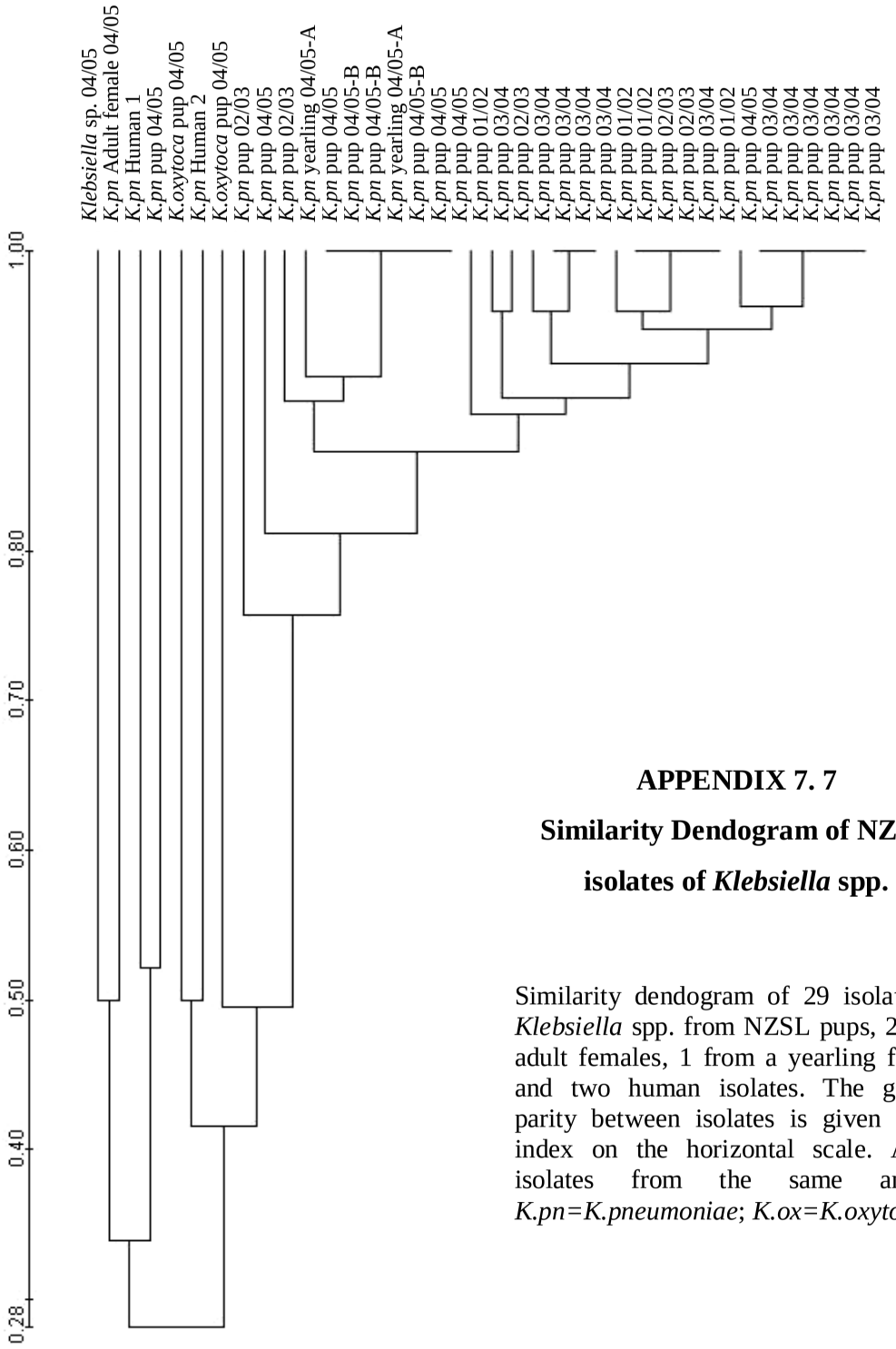
lane no.	sample
1	lambda ladder
2	*Kpn* pup 2004/2005
3	*Kpn* pup 2004/2005
4	*Kpn* pup 2004/2005
5	*Kpn* pup 2004/2005
6	*Kpn* pup 2004/2005
7	low range ladder
8	*Kpn* pup 2004/2005
9	*Kpn* pup 2004/2005
10	*Kpn* pup 2004/2005 [A]
11	*Kpn* pup 2004/2005 [A]
12	*Kpn* pup 2004/2005 [B]
13	*Kpn* pup 2004/2005 [B]
14	lambda ladder

APPENDIX 7. 6 (cont.)



A, B: isolates from the same animal

lane no.	sample
1	lambda ladder
2	*Kpn* CONTROL
3	*Kpn* adult female 2004/2005[A]
4	*Kpn* adult female 2004/2005[A]
5	*Kpn* pup 2004/2005
6	*Kpn* pup 2004/2005
7	*Kpn* adult female 2004/2005
8	*Kpn* pup 2004/2005
9	low range ladder
10	*Kox* pup 2004/2005
11	*Kpn* pup 2004/2005
12	*Kpn* Yearling 2004/2005[B]
13	*Kox* pup 2004/2005
14	*Kpn* pup 2004/2005
15	*Kpn* Yearling 2004/2005[B]
16	*Kpn* pup 2001/2002
17	lambda ladder



APPENDIX 7. 7

Similarity Dendogram of NZSL isolates of *Klebsiella* spp.

Similarity dendogram of 29 isolates of *Klebsiella* spp. from NZSL pups, 2 from adult females, 1 from a yearling female and two human isolates. The genetic parity between isolates is given as an index on the horizontal scale. A, B: isolates from the same animal. *K.pn=K.pneumoniae*; *K.ox=K.oxytoca.*

APPENDIX 9. 1

SOP for obtaining five grams of inactivated

Klebsiella pneumoniae

Principle

In order to use *Klebsiella pneumoniae* antigens in the serological test, it is necessary to obtain a large quantity of pure antigens. Its inactivation by heat reduces health risks associated with the pathogen.

Materials

Nutrient Broth preparation: 1/ Dissolve 5 grams of peptone in 850ml distilled water; 2/ Dissolve 3 grams of meat extract in the previous solution; 3/ Adjust to pH=7.0; 4/ Bring to 1L with distilled water; 5/ Autoclave.

Method

- Spread the suspension of *K. pneumoniae* contained in a frozen vial on MacConkey agar plates using a sterile cotton swab and streak the agar to obtain single colonies. Incubate the plate at 37ºC overnight.

- Inoculate two colonies into 10 ml of Nutrient Broth and incubate for 5h at 37ºC in a shaking water bath (gentle). *The culture should be saturated with a cell density of about 1-2 x 10^9 cells/mL. This can be checked with O.D. at 600 nm (0.1 O.D. = ~10^8 cells/mL).*

- Add the broth culture to a larger volume of broth (dilution of 1:10 to 1:25) in an Erlenmeyer flask (size about 5x volume of culture): total volume of 100ml in 250ml flasks and total volume of 250ml in 500ml flasks.

- Incubate the flask at 37 ºC with vigorous agitation (about 250 rpm) overnigth.

- Centrifuge the broth to pellet the bacterial cells (20min at 10, 000 rpm)

- Wash the cells twice in distilled water: spin them down and re-suspend in distilled water. Weigh the pellet.

- Heat inactivation: Suspend the pellet in 10 to 15ml of distilled water. Autoclave the bacteria for about 15 min at 121ºC in the suspension. After autoclaving, spin down again (5minutes at 3,000rpm). Freeze the pellet.

Note[1]: when autoclaving, screw the lid on but leave it loosely on the vial.
Note[2]: bacteria are now heat-killed: therefore they are not considered as infectious material anymore and can be sent by courier to another site for analysis.

APPENDIX 9. 2

SOP for reaction of various sera from New Zealand Marine Mammals with conjugates in Immunoblots

Principle

Serum samples from a range of sea mammal species are run in a Western blot (WB) test in order to determine if the chosen conjugate (protein A-G-alkaline phosphatase) will react with at least NZSL IgGs.

Reagents

10x Tris/Glycine/SDS (1L) (BioRad); High-Range Rainbow Molecular Weight Markers 250µl (Amersham/GE Healthcare Bio-Sciences); Laemmli Sample Buffer (30ml) (BioRad) with Mercapto-Ethiolate (ME); Prep Well gels 450µl (BioRad); Immunopure Protein A/G, Alkaline Phosphatase Conjugated 0.5mg (Pierce Global Science); Rabbit anti-*Klebsiella* (Jomar Diagnostics); NBT/BCIP Ready-to-use-tablets (Roche Diagnostics).

Method

Step 1 **Preparation of serum samples**

- Prepare "50µl" of each sample: 2.5µl of serum + approximately 50 µl of Laemmli sample buffer.
- "Rainbow" molecular markers: prepare 15µl of marker mix by adding 3µl of Rainbow marker to 12µlof Sample Buffer + MercaptoEthanol (ME)
- Put the 15 vials on heating platform at 95ºC for 10 minutes. Spin down for 5 minutes.
- Duration of operations: 20minutes

lane	**Sample**	**Species**	dilution	volume
1	Rainbow markers	-	1/5	5µl
2	Cetacean serum-WS00-14Kb	Pigmy Sperm whale	1/20	10µl
3	Cetacean serum-WS00-33Dd	Common dolphin	1/20	10µl
4	Pinniped serum-SS00-48Hl	Leopard seal	1/20	10µl
5	Pinniped serum-SS00-49Af	NZ fur seal	1/20	10µl
6	Cetacean serum-WS00-36Cm	Maui's dolphin	1/20	10µl
7	Cetacean serum-WB01-3Ch	Hector's dolphin	1/20	10µl
8	Cetacean serum-WS03-13Mg	Beaked whale	1/20	10µl
9	Cetacean serum-WS02-43Gm	Pilot whale	1/20	10µl
10	Pinniped serum-Ross seal (Or)	Ross seal	1/20	10µl
11	Cetacean serum SB03-02Ml	toothed Beaked Whale	1/20	10µl
12	Pinniped serum-SB03-05Ph	NZ sea lion	1/20	10µl
13	Dog serum (IDC, Wallaceville)	*Canis*	1/20	10µl
14	Sheep serum(IDC,Wallaceville)	*ovine*	1/20	10µl
15	Cow serum(IDC,Wallaceville)	*bovine*	1/20	10µl

Step 2 **Preparation of Buffer and gels**

- Running Buffer: 100ml of 10x Tris/Glycine/SDS Buffer ("for SDS PAGE"). Fill up to 1l with unsterile ELIX water (non ionized). Shake thoroughly to mix. Keep in the fridge.
Prepare 500ml of running buffer.
-Pour 250ml of Running Buffer into the electrophoresis chamber.
- Place the cooling core with the clamp assemblies or ready-gels attached into the buffer chamber. The inside of the cooling core is the upper buffer chamber; the outside is the lower buffer chamber. Hold the electrophoresis cell slightly at an angle and tap it gently to remove air bubbles from the lower end of the gels.
- Remove the comb(s) by pulling them out straight upwards. Level up the inner buffer chamber with running buffer but do not let it overflow.
- Duration of operations: 5minutes

Step 3 **Loading the samples**

- Load the appropriate volume of sample onto the gel and into the running buffer by using a pipette. The sample will flow downwards because the sample buffer contains glycerol and is heavier than the running buffer.
- For volumes 50µl and larger, slowly move the pipette tip over the whole gel length about 1-2mm above the gel surface while delivering the sample at the same time. The sample should be evenly distributed over the whole gel length.
- Duration of operations: 30minutes

Step 4 **Running the gels**

- Set at 200V (80mA) for 40 minutes.

Step 5 **Removing the gels**

Gel 1: Coomassie staining

- Cut the upper gel off. Pour Coomassie stain and leave the gel in the staining container on the rocking platform for 30 minutes.
- 2 washes with destaining solution, on the rocking platform for 10 minutes each time.
- Pour acetic acid 7% onto the gel to finalize destaining process. Leave for >10 minutes.
- Duration of operations (Gel 1): 60 minutes

Gel 2: transfer to Immobilon membrane

- TBS Buffer: already prepared. BioRad (10x) TBS 1l.
- Transfer Buffer: 100ml of 10x Tris/Glycine Buffer+ 200ml Methanol+ELIX water up to 1m. Shake it thoroughly, then open to release pressure. Keep in the fridge.
- Blocking in TBS-3%MP (Milk Powder) (without azide). Prepare 20ml of blocking solution by mixing 1.5g of milk powder with 50ml of TBS. Leave the membrane into blocking solution for 60 minutes on rocking platform.
- Wash: 3x 10minutes with TBS-0.05%Tween-20.
- Conjugate: Immunopure Protein A/G, Alkaline Phosphatase Conjugated. 1:5000. 2µl of conjugate for 10ml of TBS-3%MP.
- Wash: 3x 10minutes with TBS-0.05%Tween-20.
- Substrate: Incubate membranes with 10ml of substrate solution (dissolve 1 tablet of NBT/BCIP in 10ml of Elix water with magnetic stirrer).
- Stop: Incubate membrane in two changes of ELIX water at room temperature for 10 minutes on the rocking platform, setting 3.5. Air dry membranes and make a copy (fading).
- Duration of operations (Gel 2): 220 minutes

Results

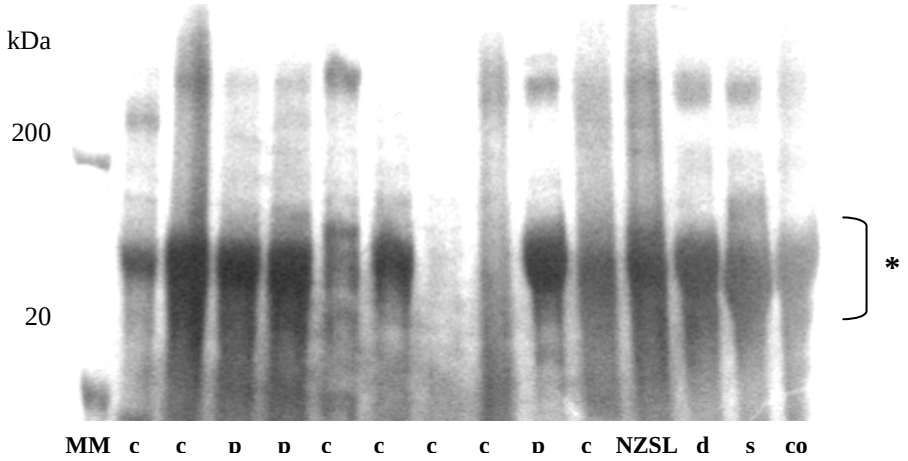


Fig. 1. Immunoblot of New Zealand marine mammal immunoglobulins reacting with the conjugate Protein A/P (*****). MM: molecular marker; c: cetacean serum; p: pinniped serum (other than New Zealand sea lion); NZSL: New Zealand sea lion serum; d: dog serum: s: sheep serum; co: cow serum.

APPENDIX 9. 3

SOP for Immunoblot reaction of *Klebsiella spp.* antigens

(*Klebsiella oxytoca* and *Klebsiella pneumoniae*)

with NZSL antibodies

Principle

To investigate the presence of circulating anti-*Klebsiella* antibodies in NZSLs, a serological test assay was developed based on Western Blotting using antigen from *Klebsiella* spp. cultured from NZSL pups that died from bacterial infection during the *Klebsiella pneumoniae* epidemics.

Materials and Reagents

- 10x Tris/Glycine/SDS (1L) (BioRad)
- High-Range Rainbow Molecular Weight Markers 250µl (Amersham/GE Healthcare Bio-Sciences)
- Laemmli Sample Buffer (30ml) (BioRad) with Mercapto-Ethiolate (ME)
- Prep Well gels 450µl (BioRad)
- Immunopure Protein A/G, Alkaline Phosphatase Conjugated 0.5mg (Pierce Global Science)
- Rabbit anti-*Klebsiella* (Jomar Diagnostics)
- NBT/BCIP Ready-to-use-tablets (Roche Diagnostics)

Method

***Day* 1, *Step 1* Preparation of antigens**

- Samples are already prepared (4.5ml of Sample Buffer (SDS/ME) + 0.5ml of bacterial solution, cf APPENDIX 9.2), heated at 95ºC for 20minutes and spun down at 10,000rpm for 15minutes. 10µl of each sample will be loaded. The samples are run in duplicates.
- Rainbow markers: prepare 6µl at 1/3 (2µl of Rainbow marker+4µl of sample Buffer+ME) and 3µl of sample buffer/ME at 1:1.
- Heat the antigen vials at room temperature before being used.

-Duration of operations: 10 minutes

lane	**Sample**	**Species**	dilution	volume
1				
2	Rainbow markers **1:1**		1:1	3µl
3	73-1	*Klebsiella pneumoniae*	1:1	10µl
4	73-2	*Klebsiella pneumoniae*	1:1	10µl
5	220-1	*Klebsiella pneumoniae*	1:1	10µl
6	220-2	*Klebsiella pneumoniae*	1:1	10µl
7	243 ox-1	*Klebsiella oxytoca*	1:1	10µl
8	243 ox-2	*Klebsiella oxytoca*	1:1	10µl
9	Rainbow markers		1:3	6µl

Day 1, Step 2 **Preparation of Buffer and gels**

- Running Buffer: 100ml of 10x Tris/Glycine/SDS Buffer ("for SDS PAGE"). Fill up to 1l with unsterile ELIX water (non ionized). Shake thoroughly to mix. Keep in the fridge.

Prepare 500ml of running buffer.

-Pour 250ml of Running Buffer into the electrophoresis chamber.

- Place the cooling core with the clamp assemblies or ready-gels attached into the buffer chamber. The inside of the cooling core is the upper buffer chamber; the outside is the lower buffer chamber. Hold the electrophoresis cell slightly at an angle and tap it gently to remove air bubbles from the lower end of the gels.

- Remove the comb(s) by pulling them out straight upwards. Level up the inner buffer chamber with running buffer but do not let it overflow.

- Duration of operations: 5minutes

Day 1, Step 3 **Loading the samples**

- Load the appropriate volume of sample onto the gel and into the running buffer by using a pipette. The sample will flow downwards because the sample buffer contains glycerol and is heavier than the running buffer.

- For volumes 50µl and larger, slowly move the pipette tip over the whole gel length about 1-2mm above the gel surface while delivering the sample at the same time. The sample should be evenly distributed over the whole gel length.

- Duration of operations: 20minutes

Day 1, Step 4 **Running the gels**

- Set at 200V (80mA) for 40 minutes.

Day 1, Step 5 **Removing the gels**

Day 1, Step 6 **Transfer onto Immobilon membranes**

- TBS Buffer: already prepared. BioRad (10x) TBS 1l.

- Transfer Buffer: 100ml of 10x Tris/Glycine Buffer+ 200ml Methanol+ELIX water up to 1m. Shake thoroughly, then open to release pressure. Keep in the fridge.

- Place the membranes into Transfer Buffer.

- Dip the 2 membranes into methanol then into transfer buffer; dip the 4 filter papers into transfer buffer. Place the filter papers onto the semi dry blotter platform (get rid of air bubbles with a roll), then the two membranes (gently use a roll to avoid air trapped), then the gels, and finally the last layer of filter paper. Lock the cover of the blotter. Apply 15V for 30 minutes.

- Pour transfer buffer into boxes to re-moist membranes!

- Duration of operations: 40 minutes

Day 1, Step 7 **Blocking**

- Block in TBS-3%MP (Milk Powder) (without azide). Prepare 50ml of blocking solution by mixing 1.5g of milk powder with 50ml of TBS. Leave the membrane into blocking solution OVERNIGHT on rocking platform.

- Duration of operations: 10minutes+overnight

***Day* 2, *Step 1* Incubation with Rabbit Antibodies**

- Wash: TBS-0.05%Tween-20. Once for 10 minutes on rocking platform.
- Prepare Antibodies solutions in 15ml red top PP-test tubes: A (1:1000) 10µl of Ab with 10ml of freshly prepared TBS-MP3%/ B (1:2000) 5µl Ab with 10ml TBS-MP3%/ C (1:4000) 2.5µl Ab with 10ml TBS-MP3%/ D (1:8000) 1.25µl Ab with 10ml TBS-MP3%.
- Heat in 37C incubator for 5minutes maximum. Vortex.
- Pour the TBS-Tween off the boxes. Cut the membranes into halves and place them into the 4 boxes A, B, C and D.
- Pour the Antibodies solutions in their respective box.
- Incubate at RT for 60 minutes (between 18 and 28C).
- Wash: TBS-0.05%Tween-20 three times for 10 minutes each.

-Duration of operations: 80minutes

***Day 2, Step 2* Reaction with Conjugate**

- Conjugate: Immunopure Protein A/G, Alkaline Phosphatase Conjugated. 1:5000. 2µl of conjugate for 10ml of TB-Tween.
- Leave for 60 minutes at RT.
- Wash: 3x 10minutes with TBS-0.05%Tween-20.

-Duration of operations: 95 minutes

***Day 2, Step 3* Reaction with Substrate**

- Substrate: Incubate membranes with 10ml of substrate solution (dissolve 1 tablet of NBT/BCIP in 10ml of Elix water with magnetic stirrer).
- Stop: Incubate membrane in two changes of ELIX water at room temperature for 10 minutes on the rocking platform, setting 3.5. Air dry membranes and make a copy (fading).
- Duration of operations: 15 minutes

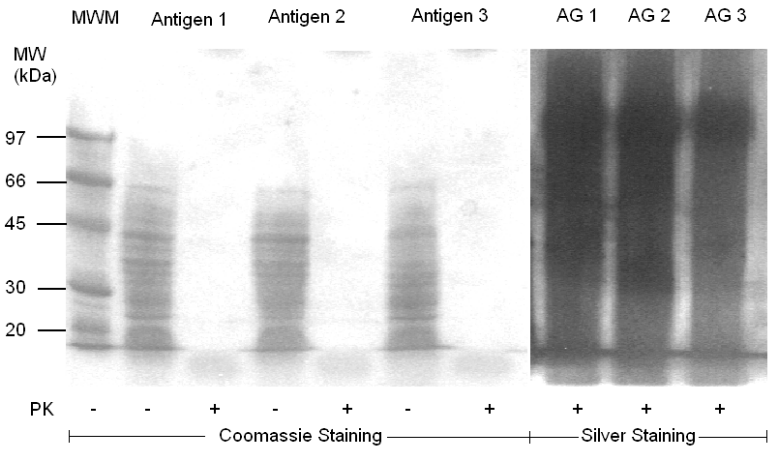


Fig. 1. Analysis of various *Klebsiella* antigen preparations in polyacrylamide gels, by a combination of proteinase K (pK) digestion and Coomassie- and silver-staining.

APPENDIX 9.4

Electrophoretic immunoblotting-

Detection of anti-*Klebsiella* antibodies

in NZSL sera

Principle, Materials and Reagents: see APPENDIX 9.3

Methods

***Day* 1, *Step 1* Preparation of antigens**

- rainbow markers: prepare twice 6µl at 1/3 (2µl of Rainbow marker+4µl of sample Buffer+ME).
- supernatant of the antigen (*K.pneumoniae* had been incubated with 40µl of ProteinaseK at 56ºC for 1 hour)
- gel: 4-15% Tris-HCl 2D/Prep Comb (BioRad, New Zealand)
- Duration of operations: 10 minutes

Wells #	Solution	dilution	volume
1	Rainbow markers 1:3	1:3	6µl
2	Antigen supernatant (pK digested)	-	100µl

***Day 1, Step 2* Preparation of Buffer and gels**

- Running Buffer: 100ml of 10x Tris/Glycine/SDS Buffer ("for SDS PAGE"). Fill up to 1 litre with unsterile ELIX water (non ionized). Shake thoroughly to mix. Keep refrigerated.
- Pour 250ml into the electrophoresis chamber.
- Place the cooling core with the clamp assemblies or ready-gels attached into the buffer chamber.
- Hold the electrophoresis cell slightly at an angle and tap it gently to remove air bubbles from the lower end of the gels.
- Remove the comb(s) by pulling them out straight upwards. Level up the inner buffer chamber with running buffer but do not let it overflow.
- Duration of operations: 10 minutes

***Day 1, Step 3* Loading the samples**

- Load the appropriate volume of sample onto the gel and into the running buffer by using a pipette (the sample will flow downwards because the sample buffer contains glycerol and is heavier than the running buffer).
- Slowly move the pipette tip over the whole gel length about 1-2mm above the gel surface while delivering the sample at the same time.

Note: For this particular gel, the comb for the antigen takes the whole width of the gel (with a small comb for the markers). The sample should be evenly distributed over the whole gel length.

- Duration of operations: 5 minutes

Day 1, Step 4 **Running the gel**

- 200V (80mA) for 40 minutes.

Day 1, Step 5 **Removing the gels**

Day 1, Step 6 **Transfer**

- TBS Buffer (10xTBS 1litre).
- Transfer Buffer: 100ml of 10x Tris/Glycine Buffer + 200ml Methanol+ELIX water up to 1 litre. Shake thoroughly then open to release pressure. Keep refrigerated.
- Dip the Immobilon© membrane into methanol then into transfer buffer.
- Dip the 2 filter papers into transfer buffer. Place the filter paper onto the semi dry blotter platform (get rid of air bubbles with a roll), then the membrane (gently use a roll to avoid air trapped). Place the gel on top of the membrane, and finally the last layer of filter paper.
- Lock the cover of the blotter.
- Apply 15V for 30 minutes.
- Pour transfer buffer into boxes to re-moist membrane.

-Duration of operations: 35 minutes

Day 1, Step 7 **Blocking**

- Blocking solution: prepare 50ml of blocking solution by mixing 1.5g of milk powder with 45ml of ELIX water and 5ml of {TBS+Merthiolate 1/1000^{e}}.
- Block in TBS-3%MP (Milk Powder) (without azide).
- Leave the membrane into blocking solution OVERNIGHT on rocking platform.
- Duration of operations: 10 minutes + overnight

Day 2, Step 1 **Preparation of standards**

Lane	Sample	Dilution
5	*Control +*	Antisera 1:5 000
6	*Control +*	Antisera 1:20 000
7	*Control +*	Antisera 1:80 000
8	*Control -*	New Zealand sea lion pup #E00/01-5Ph 1:200

Day 2, Step 2 **Assembling the miniblot cassette system**

The membrane is now divided into single lanes by using the Immunetics® Cassette Miniblot System. Refer to the Immunetics Cassette Miniblot System Instructions for more detailed information on this system.

- Turn the cassette over and with forceps take the membrane out of the incubation tray and let the blocking solution trip off. Align the membrane precisely against the channels (according to the

pencil markings), with the antigen bearing face of the membrane facing the channels. Make sure that the membrane covers the full length of each channel in the cassette.
- Turn the Miniblot C-shell front plate face down and insert the cassette with the membrane on its back into the recessed back of the front plate. The entry ports of the cassette fit into the horizontal slots of the C-shell.
- Place the cushion over the membrane, and ensure that it covers the entire channel pattern on the cassette.
- Set the back plate of the C-shell on the inverted front plate with the cassette and membrane, so that the alignment pins fall into place. Turn the entire C-shell over and insert both screws, tightening gently and equally as far as they will turn by hand. Do not tighten excessively!
- Aspirate excess liquid from the channels through the numbered entry ports by using a disposable pipette tip, which is connected to a water vacuum aspirator. To avoid excessive drying of the membrane, channels should be loaded directly after this step.
- Duration of operations: 10 minutes

Day 2, Step 3 Loading of samples and incubation

- The channels are numbered.
- Dilute the sera in TBS-MP. Dilution 1:200 (1microlitre of serum into 200 μl of TBS-MP)
Introduce 50μl of diluted serum through the entry port with a pipette, using standard disposable plastic pipette tips. Press the pipette tip straight and firmly into each entry port and inject liquid smoothly and rapidly until the channel is filled. There are 28 channels per cassette.
Start filling from channel 5 to leave enough space for the molecular markers on the left of the membrane. All unused channels and the channels covering the molecular weight standards are filled with TBS-MP.
- Incubate the cassette on the rocking platform at 5-6 tilts per minute for 2 hours at room temperature.
- Wash once with 100ml of TBS-Tween per membrane with the Miniwash© manifold. To aspirate all samples simultaneously, connect one side of the manifold to the water jet vacuum source and the other side to a bottle containing TBS-Tween.
- Remove the membrane from the MINIBLOT C-Shell and wash it twice in 20ml TBS-Tween in a tray for 10 minutes on the rocking platform at 15-30 tilts per min. (setting 3½).
- Duration of operations: 140 minutes

Day 2, Step 4 Detection system: reaction with conjugate-substrate

- Incubate the membrane in a tray in 10ml of appropriate conjugate solution, diluted in TBS-MP at the appropriate dilution. Incubate by agitating on a rocking platform at 15-30 tilts per min. (setting 3½ for Immunetics Rocker) for 30 min. at room temperature.
- Discard the conjugate solution.

- Wash the membrane 3 times with 20ml TBS-Tween for 10min. on the rocking platform at 15-30 tilts per min. (setting 3½).
- Duration of operations: 65 minutes

Day 2, Step 5 Incubation with substrate

- Incubate the membrane in a tray with 10ml of substrate (NBT/BCIP) solution for 3 min. (or according to individual protocols) on the rocking platform at 15-30 tilts per min. (setting 3½) at room temperature.
- Then discard the substrate.
- Wash three times with 20ml ELIX water for 10 min. on the rocking platform.
- Transfer the membrane onto a plastic board and let it air-dry. Some background staining may appear during the substrate reaction parallel with the specific staining but disappears usually during drying, while the specific staining remains.
- Duration of operations: 3 minutes

Note: Hazard: NBT/BCIP substrate is toxic and possibly carcinogenic. Wear gloves.

Step 6 Interpretation of test

Stained bands of individual sera on the immunoblot are compared to the positive control serum. Depending on the staining pattern, a sample is classified as negative, positive + score 1, ++ score 2, +++score 3.

On the following page: **Fig 1.** Electrophoresis assembling ; **Fig 2.** Immunetics® Cassette Miniblot; **Fig 3.** Rocking platform; **Fig 4.** Rinsing the membranes with TBS-Tween (bottle) using the Miniwash© manifold. To aspirate all samples simultaneously, connect one side of the manifold to the water jet vacuum source and the other side to a bottle containing TBS-Tween. **Fig 5.** Stained bands with the Rainbow molecular weight markers on the left.

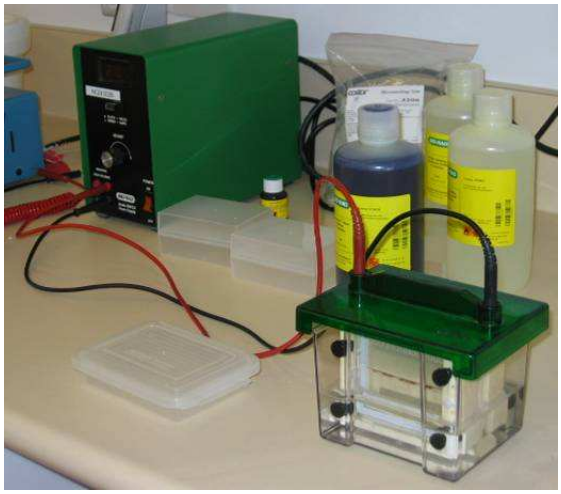


Fig 1.

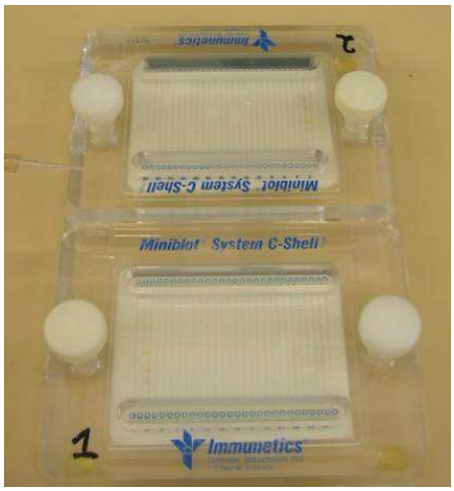


Fig 2.

Fig 3.

Fig 4.

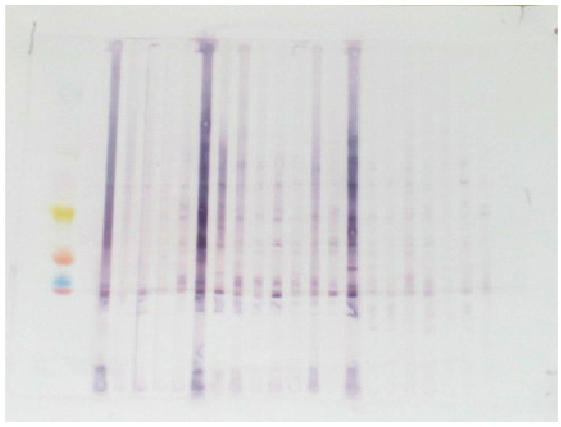


Fig 5.

APPENDIX 9.5

SOP for Purification of Immunoglobulins from serum of NZSLs by Ammonium Sulphate (AS) precipitation

Principle:
Immunoglobulins will precipitate out of serum at around 50% saturated AS, while most other proteins will stay in solution (at 25C, a saturated AS solution is 4.1M).

Materials
- Bovine Gamma globulin (BGG) 2mg/ml (Biorad)
- Coomassie Stain solution (1L bottle) (BioRad)
- Destain Solution, Coomassie R-250 (1L bottle) (BioRad)
- *see* APPENDICES 9.2, 9.3 and 9.4 for other reagents.

Method
- Centrifuge the serum at 4,400rpm for 15 minutes at 4C. For 10ml of serum supernatant add 3.1 g solid AS (*4.5ml so add 1.4g of AS)* + 5microL of 10% merthiolate (1/1000) to prevent micro-organisms from growing. Keep stirring at 4C for 1-4hours or overnight

Note: the solid AS (salt) should become completely dissolved, while the IgGs appear as a fluffy precipitate.

- Centrifuge the solution at 2000-4000g for 15-20 min at 4C
- Carefully remove the supernatant
- Drain the pellet by carefully inverting the tube over a paper filter
- Dissolve the precipitate in 10-20% of the original volume with TBS, with a spatula or repeatedly drawing into wide-gaged Pasteur pipette
- Dialyse extensively (several buffer changes) against TBS at 4C overnight
- Determine the protein amount (Bradford method, Quantity One © BioRad software) and check purity by PAGE (Coomassie stain).

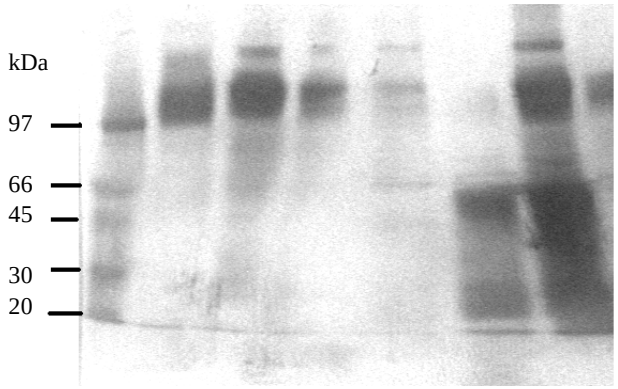


Fig. 1. Verification of the degree of purity of IgGs from adult female NZSLs by SDS-PAGE (Coomassie stain).

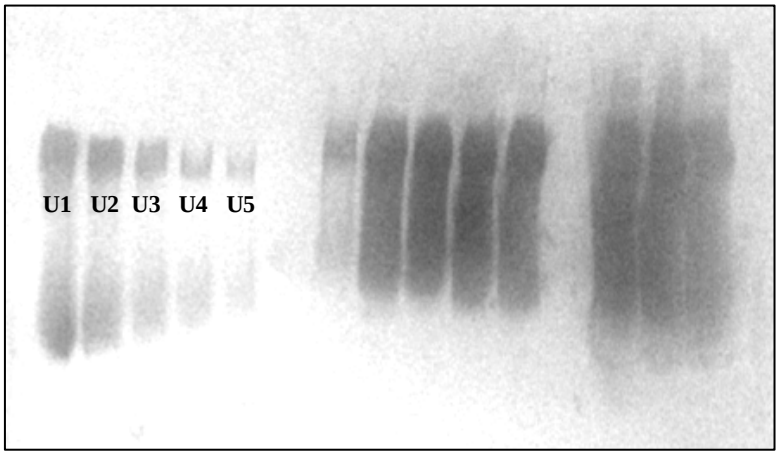


Fig.2a.

Index	Name	Density (OD)	Conc mg/ml
1	U1	1188.18780	1.6
2	U2	1117.36815	0.8
3	U3	1036.72316	0.4
4	U4	967.54068	0.2
5	U5	935.01338	0.1

Fig.2b.

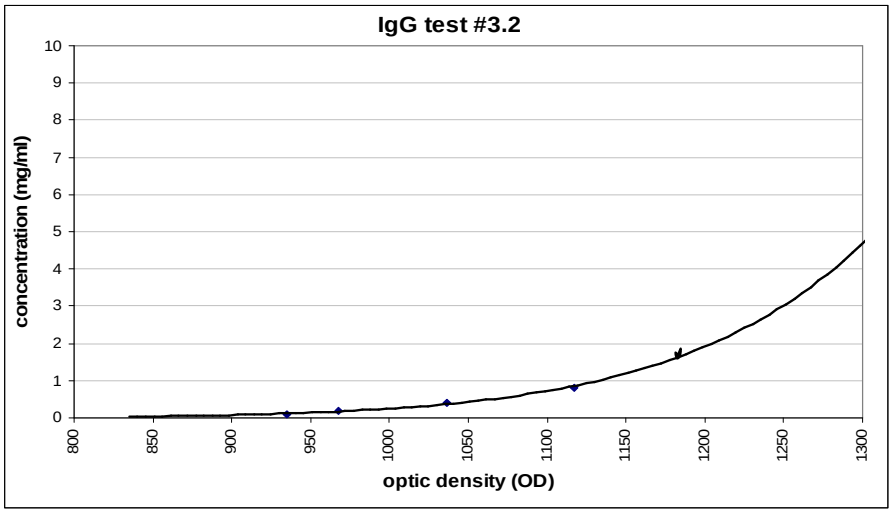


Fig.2c.

Fig.2. Determination of the total IgG concentrations in NZSL serum (Test 3.2) (Fig.2a) using the Bradford method & Quantity One© (BioRad) software (Fig.2b). Optic densities (OD) are plotted against known Ig concentrations. Ig concentrations are graphically determined using measured ODs (Fig.2c).

APPENDIX 9.5 (cont.)

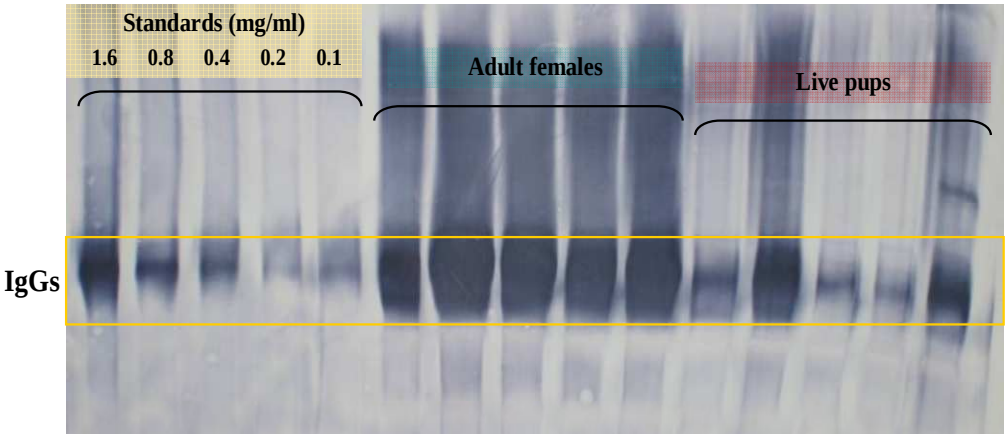


Fig. 3. Examples of IgGs from NZSL adult females and pups.