Assessing the impacts of infectious disease on reproductive success in New Zealand sea lions (*Phocarctos hookeri*)

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Abstract

Poor reproductive success is one factor that may be perpetuating the population decline of the threatened New Zealand (NZ) sea lion (*Phocarctos hookeri*). The species has a severely restricted distribution, with 99% of breeding occurring on the remote NZ sub-Antarctic islands and amongst the lowest expected reproductive outputs compared to other otariids. Infectious disease, particularly septicaemia caused by the bacterium *Klebsiella pneumoniae* is known to be a major mediator of early pup mortality, but the role of infectious disease in impairment of reproductive success has not been investigated.

This thesis aimed to fill this knowledge gap by investigating three areas of concern. Firstly, the role of infectious disease in stillbirth of NZ sea lion pups was examined with a histopathological study of archived necropsy tissues. Secondly, the seroprevalence of adult and juvenile NZ sea lions to *Toxoplasma gondii*, a known cause of reproductive failure, at several locations was evaluated. Finally, a survival analysis was conducted to model the long term survival and reproductive success of pups that were treated with ivermectin as pups, to assess ongoing benefits of early hookworm burden removal.

In contrast to the mass mortalities seen with bacterial disease in NZ sea lion colonies, at least in the topics covered in this thesis, the role of infectious disease contributing to poor reproductive success is apparently minimal. No specific infectious agents were identified to have caused the death of the stillborn pups examined, however pneumonia was diagnosed in four animals. A low seroprevalence to *T. gondii* was found in mainland but not sub-Antarctic colonies, however those animals with strongly positive titres showed no clinical signs and had reproduced normally. Finally, although the survival analysis was limited by small sample size and very poor juvenile survival, it depicted promising trends for improved survival for those pups treated with ivermectin as pups. All studies have generated areas for future research and recommendations for further conservation management of this vulnerable species.

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lions on the mainland and sub-Antarctic between 1998 and 2012 for sample collection and resighting records.

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Baltic ringed seal	*Pusa hispida*
Bottlenose dolphin	*Tursiops truncatus*
California sea lion	*Zalophus californianus*
Grey seal	*Halichoerus grypus*
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South African fur seal	*Arctocephalus pusillus*
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Subantarctic fur seal	*Arctocephalus tropicalis*

Chapter One. Literature Review

1.1. Introduction

New Zealand (NZ) sea lions (formerly Hooker's sea lion, *Phocarctos hookeri*) are large pinnipeds of the family *Otariidae* (eared seals: fur seals and sea lions). A hallmark of this family is marked sexual dimorphism, with dark brown NZ sea lion males reaching weights over 400kg, more than twice that of the creamy grey females (Figure 1.1; Walker and Ling, 1981). The species is highly gregarious and polygynous (Marlow, 1975), and where populations are large enough, NZ sea lions breed in colonies. Females are strongly philopatric to their natal colony and will return there to breed once mature (Chilvers and Wilkinson, 2008). Males on the other hand are known to disperse to the limits of the species' range and breeding males may move between colonies within a breeding season (Robertson et al., 2006).

Figure 1.1 New Zealand sea lions (*Phocarctos hookeri*) at Sandy Bay, Enderby Island.A large dark brown adult male (left), creamy grey adult females (foreground) and a pup (right) are seen in this harem.

The NZ sea lion is the only pinniped endemic to New Zealand. The species is classified as 'nationally critical' by the New Zealand threat classification system and 'threatened' by the International Union for the Conservation of Nature due to small population size and limited distribution (Gales, 2008, Baker et al., 2010). NZ sea lions were historically present along the coasts of both the North and South Islands of New Zealand but following years of exploitation, their breeding range is now restricted primarily to the New Zealand sub-Antarctic islands between 50-53°S, on the Auckland Islands and Campbell Island (Figure 1.2; Childerhouse and Gales, 1998). In the last 25 years however there has been very slow recolonisation of the mainland at the Otago Peninsula and adjacent Catlins coast on the South Island, as well as Stewart Island (McConkey et al., 2002b, Chilvers et al., 2007b).

1.2. Population dynamics

1.2.1. *Auckland Islands*

The Auckland Islands (Figure 1.2) form the centre of the highly localised breeding range for the NZ sea lion, with approximately 73% of breeding occurring within two colonies on islands less than 10km apart (Maloney et al., 2012). The primary breeding site is Dundas Island (50°35'S, 166°19'E; Figure 1.3a), a tiny two hectare island where over 50% of pupping takes place (1213 pups in 2013-14; Childerhouse et al., 2014). Sandy Bay, Enderby Island (50°30'S, 166°17'E; Figure 1.3b) is the second largest breeding colony of the NZ sea lion and due to relative ease of access has been the most intensively studied. These islands together are considered to be a single 'breeding area' due to close proximity and continual interchange of breeding males within a breeding season (Robertson et al., 2006). There is some additional breeding at Figure of Eight Island (50°46'S, 166°01'E; Figure 1.3c) in Carnley Harbour at the south of the Auckland Island group, however pup production has halved over the last two decades, down to 72 pups counted in the 2013/14 season (Childerhouse et al., 2014).

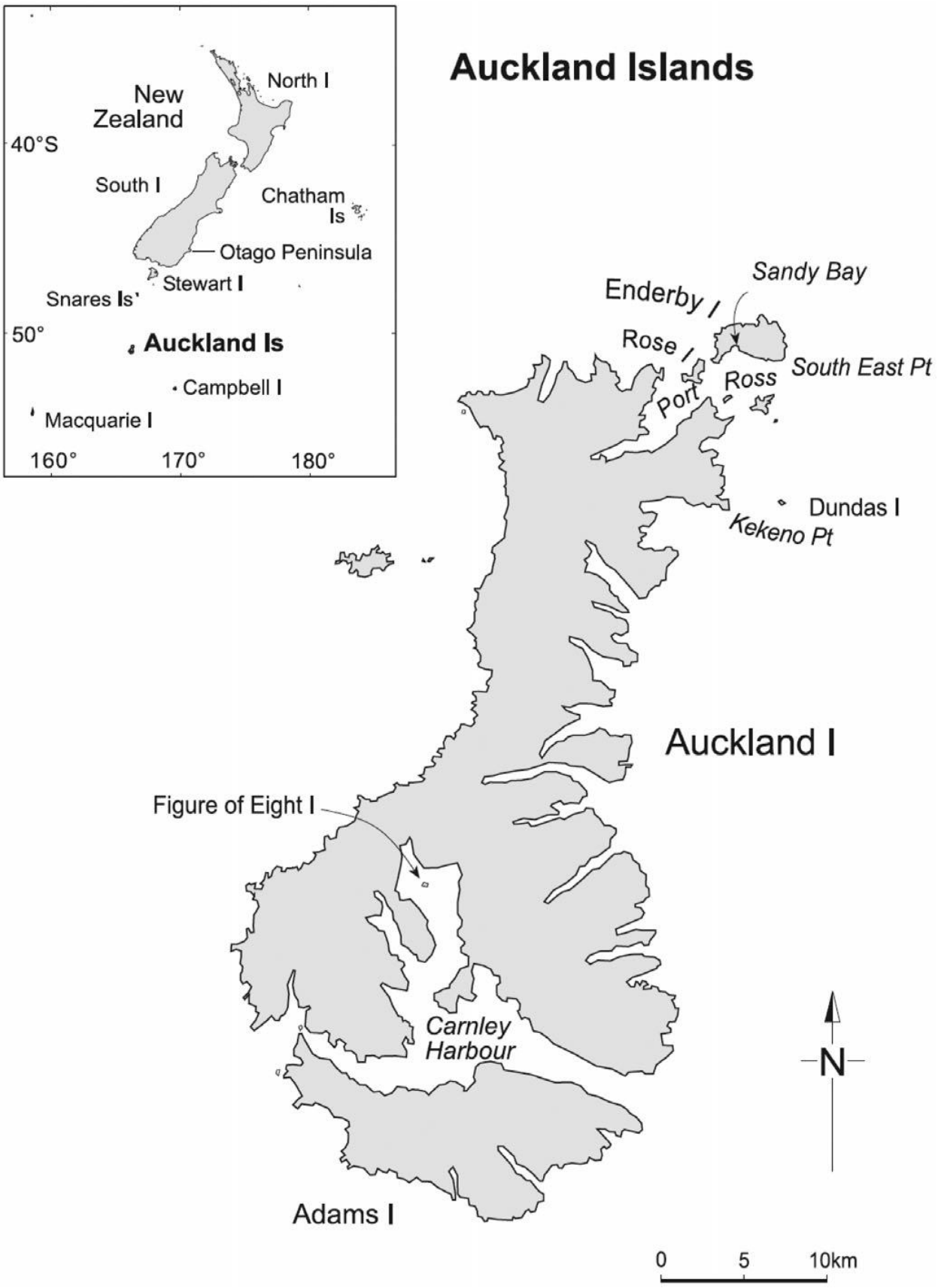

Figure 1.2 Map of New Zealand and sub-Antarctic islands showing the primary NZ sea lion (*Phocarctos hookeri*) breeding areas of Sandy Bay, Enderby Island; Dundas Island; Figure of Eight Island and Campbell Island. Image credit: Department of Conservation, New Zealand.

Figure 1.3 New Zealand sea lion (*Phocarctos hookeri*) breeding colonies at the Auckland Islands showing A. Dundas Island; B. Sandy Bay, Enderby Island and C. Figure of Eight Island. Dundas Island aerial photo credit: D Donnelly, Figure of Eight Island aerial photo credit: BL Chilvers.

Following discovery of the Auckland Islands in 1806, local pinnipeds were exploited for their skins and flesh. Initially NZ fur seals were targeted, but as that population dwindled, sealers set their sights on the less highly prized skins of NZ sea lions, with large numbers killed. Throughout the 1800s the NZ sea lion population at the Auckland Islands appears to have oscillated between near depletion and recovery with several periods of sealing, before official protection of the species was instigated in 1893 (Childerhouse and Gales, 1998).

The Sandy Bay colony has been well studied, with regular population estimates undertaken each austral summer consistently since 1995 (Gales and Fletcher, 1999). In pinnipeds, pup production is the best index of relative population status and when combined with other population parameters, forms an indicator of overall population size and trends (Berkson and Demaster, 1985). Additionally, pups are ideal to work with in the field, as they are restricted to land, hence much easier to identify and count than adults that are highly vagile (Department of Conservation, 2009). Initial trends showed increasing pup production at the Auckland Islands between 1995 and 1998. However, since 1998 pup production has declined by almost 50%, reflecting a decline in overall population size (Chilvers, 2012, Childerhouse et al., 2014). Potential causes for this continuing decline have been proposed including interaction with fisheries (resource competition and by-catch) as well as bacterial epizootics (Robertson and Chilvers, 2011).

Fisheries interactions with NZ sea lions in the sub-Antarctic have been reported to negatively affect populations through direct impacts causing mortality of adult animals, as well as indirect effects of resource competition. Diet analysis and foraging studies have established that there is overlap between preferred Auckland Island NZ sea lion prey items and feeding grounds with the SQU6T arrow squid (*Nototodarus sloanii*) fishery (Chilvers, 2008, Meynier et al., 2009). Compared to mainland populations, sub-Antarctic females have been shown to regularly reach their physiologic limits while foraging in order to meet the energy demands of themselves and often a dependent pup ashore (Chilvers et al., 2006). This kind of 'nutritional stress', here imposed by competition for prey

and low nutritional content of available prey has been proposed to decrease reproductive success in Steller sea lions (*Eumetopias jubatus*; Trites and Donnelly, 2003) and a similar effect may occur in NZ sea lions. Additionally, bycatch mortality of NZ sea lions within this fishery has previously been nominated as the largest anthropogenic impact on the species, with 234 NZ sea lions caught on observed vessels between 1992 and 2009 (Robertson and Chilvers, 2011). Of those animals, 71% were female, with implications of downstream effects causing starvation of potential dependent pups (Robertson and Chilvers, 2011). Within the last decade, sea lion exclusion devices have been deployed within the Auckland Island fishery to allow escape of captured animals through a hatch at the top of the net, although their effectiveness is controversial and evaluation is ongoing.

Bacterial epizootics have occurred on three occasions at the Auckland Islands population since regular summer monitoring has taken place, accounting for significant losses of pups (Table 1.1) and therefore decreased recruitment of breeding females into the population three to seven years after each event (Wilkinson et al., 2004).

Table 1.1. Summary of known New Zealand sea lion (*Phocarctos hookeri*) epizootic events at the Auckland Islands.

Season	1997/98	2001/02	2002/03
Causative agent	Gram negative pleomorphic bacteria consistent with *Campylobacter* spp.	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
Known sites affected	Enderby and Dundas Islands	Enderby Island	Enderby Island
Pup mortality rate*	52.6%	33%	21.2%
Adult mortality	74 adult females	-	-
Reference	(Baker, 1999, Roe, 2009)	(Wilkinson et al., 2004)	(Wilkinson et al., 2004)

*Pup mortality rate in monitored summer field season (December to mid-February)

The first known epizootic was the most devastating for the population, occurring in January and February of 1998 and involving Enderby and Dundas Islands. An estimate of 1596 pups (52.6%) died within the period monitored (December to mid-February), often in good condition with no observed clinical signs (Baker, 1999). The mortality rate for Dundas Island was three times higher than normal

for that stage of the season. Due to limited resources, necropsy and sample collection of only a small proportion of affected animals was obtained.

Post mortem and histopathology of 13 pups from the event showed a range of lesions including pneumonia, suppurative stifle arthritis, suppurative encephalitis and suppurative lymphadenitis, however many had no gross or histopathological lesions (Duignan, 1999). Findings in adults included abscessation of the throat area, multifocal, well-defined, raised swellings of the skin on the ventrum, or apparent hind limb paralysis, which may have been secondary to lumbar abscessation, although few gross necropsies were undertaken (Duignan, 1999). Histopathology of tissues from seven adult females showed fulminating septicaemia and vasculitis. A member of the *Campylobacter* genus was identified on 16S rRNA sequencing, but was difficult to sustain in culture, so no further investigation was undertaken (Roe, 2009). Algal biotoxins, contaminants including organochlorines, unusual climatic events and viral disease were investigated and concluded to be unlikely causes of the mortality event.

Cumulative totals of 33% and 21.2% of pups died in the monitored period (December to February), in the two summer seasons between 2001 and 2003, with mortality rapidly increasing from the third week of age in mid-January (Wilkinson et al., 2004). Affected pups showed clinical signs of fluctuant swellings of the limbs, progressive lameness, lethargy and convulsions. Necropsies were conducted on 243 pups over the two seasons. The most common findings were suppurative, necrotising arthritis, tenosynovitis and cellulitis of the carpus and tarsus, often extending proximally up the affected limb(s). Suppurative arthritis of the atlanto-occipital joint was also commonly seen as well as meningitis, peritonitis and pleuritis. *Klebsiella pneumoniae* was isolated in pure culture from multiple organs of 83% of pups diagnosed grossly as having bacterial infection. Further investigation has shown that both mortality events were due to genetically indistinguishable isolates of *K. pneumoniae*, suggesting a single introduction of a novel highly pathogenic strain of the bacteria to a naïve population (Castinel et al., 2007c).

In addition to mass neonatal mortality, greatly decreased numbers of pups were born in 2001/02 with 30% and 20% lower estimates of pup production than the previous year at Sandy Bay and Dundas Island respectively (Wilkinson et al., 2004). From analysis of counts of females ashore and pup to cow ratios, it was concluded that low pup production was a result of decreased fecundity, rather than poor return rate of females to give birth, suggesting subclinical effects of the epidemic on breeding females (Wilkinson et al., 2004).

The only other significant negative deviation in pup production at the Auckland Islands was in the 2008/09 season. Estimates were 31% lower than the previous year, establishing the lowest pup production recorded, with only 1501 pups born. This has been attributed to significantly decreased return of breeding females to the colonies, although the reason for this is unknown (Robertson and Chilvers, 2011).

Neonatal mortality has been studied at Sandy Bay by Castinel et al. (2007b) throughout the summer seasons between 1998 and 2005, showing a mean of 10.2% pup mortality in non-epidemic years. Mortality was categorised into the most commonly identified cause of trauma (32.2%) followed by starvation (17%), hookworm infestation (16.4%) and bacterial infections (13.5%). More recent research from the 2007/08 season however has shown that a significant proportion of deaths diagnosed at gross necropsy to be caused by head trauma, with signs of subdural haemorrhage, were in fact meningitis caused by *K. pneumoniae* (Roe, 2012). Consequently, more research is required in this area and infectious disease should be looked at more closely in terms of pup loss, as *K. pneumoniae* may persist as an important cause of mortality in non-epizootic years. Stillbirth was present at a low rate each year causing an average of 4.2% of deaths regardless of epizootics and congenital deformities also had low prevalence at 2.6%.

1.2.2. ***Campbell Island***

Campbell Island (Motu Ihupuku; 52°33'S, 169°09'E; Figure 1.4) represents the most extreme southern limit of the NZ sea lion breeding range and is the only significant breeding area outside of the Auckland Islands. There have been limited population estimates in the Campbell Island NZ sea lion population since the discovery of the island in 1810, with initial population numbers prior to human exploitation unknown. Following early settlement, populations of fur seals and New Zealand sea lions were quickly decimated by the commercial sealing industry, mainly harvesting the animals for their skins (McNally et al., 2001). After twenty years of exploitation, the sealers withdrew due to

Figure 1.4 Map of Campbell Island/Motu Ihupuku showing primary New Zealand sea lion (*Phocarctos hookeri*) breeding sites including Davis Point and Paradise Point. Image credit: Department of Conservation, New Zealand.

declining profits and the sea lion population was gradually able to recover. Between 1909 and 1916, a whaling station based at Northwest Bay regularly killed sea lions for dog food, again reducing the population, at least locally, to low numbers (Timms et al., 1978). Within the last century estimates have been varied and inconsistent, due to opportunistic counts, differing methodologies and logistical problems associated with rugged terrain and harsh weather conditions.

The primary breeding colony is an exposed basalt platform at Davis Point (Figure 1.4), a peninsula on the east coast of Campbell Island, on the northern shore of the entrance to Perseverance Harbour. Until 2008, this was the only known area where pups, cows and bulls have been found together, although historically its use has been sporadic. A second smaller aggregation of breeding females was discovered in the 2007/08 field season at Paradise Point (Figure 1.4), on the southern side of Perseverance Harbour, consisting of an exposed slope with a narrow rocky shore. Outside of these colonies, breeding behaviour is non-colonial, with pupping taking place inland, amongst dense vegetation. Pups have been found widely dispersed over Campbell Island, including 1.4km inland and up to 400m in altitude (Maloney et al., 2009). Consequently, the difficulty in assuring that all animals are counted in rugged terrain and severe weather conditions that can limit visibility to less than one metre is a significant and largely unavoidable source of error in any of these population estimate surveys.

Estimates of minimum total pup production have generally increased over time, with more recent studies undertaking more geographically extensive surveys over longer field seasons (Table 1.2). Further research is required to develop easily comparable methods of estimating population to fully assess population dynamics in this site.

Pup mortality rate has been consistently high on Campbell Island and most deaths have been identified at Davis Point, consistent with the known distribution of the population and difficulty finding dead pups away from the coast. When gross necropsies have been carried out, they have included only a limited sample and no correlating histopathology has been undertaken to confirm

Table 1.2. Summary of recent New Zealand sea lion (*Phocarctos hookeri*) pup population estimates on Campbell Island.

Season	1991-92	1997-98	2002-03	2007-08	2009-10
Minimum total pup production	122	78	385	583	681
Davis Point	-	54	166	442	503
Paradise Point	-	-	-	122	168
Pup mortality rate*	-	44%	36%	40%	55%
Length of field season	opportunistic	44 days	58 days	48 days	64 days
Method of estimate	Direct count	Direct count, strip transects	Mark – recapture	Direct count	Direct count
Reference	(Gales and Fletcher, 1999)	(McNally et al., 2001)	(Childerhouse et al., 2005)	(Maloney et al., 2009)	(Maloney et al., 2012)

*Pup mortality rate within monitored field season period

field diagnoses of cause of death. Trauma, malnutrition, hookworm infestation, bacterial infections, exposure, drowning in peat mires or a combination of these have been reported as cause of mortality (Maloney et al., 2009). A consistent and common finding in live pups has been superficial to deep ulceration of the ventral carpus of the fore flippers or various parts of the hind flippers (Maloney et al., 2009). It is likely that this is secondary to abrasion from the basalt substrate of the Campbell Island colonies, but this damage can lead to infection and poor mobility, therefore increasing the risk of death by crushing and trauma. Tissues were collected from 15 dead pups in the 2008/09 season with gross lesions suggestive of bacterial infection. *K. pneumoniae* was not cultured from collected tissues, however 60% (9/15) of these samples grew *Streptococcus* spp. on microbiological culture (Maloney et al., 2009). No stillbirths have been reported at Campbell Island, but only one expedition has been present at height of pupping in mid to late December (Maloney et al., 2012).

1.2.3. ***Otago***

The Otago region on the south-eastern coast of the South Island, New Zealand is the current northernmost limit of year-round presence by NZ sea lions, concentrated at the Otago Peninsula (46°S, 170°40'E) and Catlins coast (46°30'S, 169°30'E) regions. Subadult males have long been

observed to haul out in these locations but recolonisation by breeding females has only occurred recently with a single founder female (tagged as a pup at the Auckland Islands in the 1986/87 breeding season) pupping on the Otago Peninsula for the first time in 1992 (McConkey et al., 2002b). This was unusual for the species, which are usually highly philopatric to their natal site (Chilvers and Wilkinson, 2008). Surviving offspring from the founder female have shown site fidelity to the Otago region and have since developed a small population, based on the original matriarchal line, with around five pups born annually. The total number of breeding females is less than 0.1% of the total breeding population, therefore Otago is not currently recognised as a breeding area (Chilvers et al., 2007b, Robertson and Chilvers, 2011).

1.2.4. ***Stewart Island***

Historically Stewart Island (47°S, 168°E), off the southern coast of the South Island of New Zealand has been a regular haul out location for subadult males (Wilson, 1979). Between 1988 and 2001, only five pups were born on Stewart Island, reflecting inconsistent breeding (McConkey et al., 2002b). Surveys since 2011 have reported between 16 to 32 pups born annually, making this the largest breeding area outside of the sub-Antarctic, although still contributing less than 1% of the species' total pup production (BL Chilvers pers. comm).

1.2.5. ***Snares Islands***

NZ sea lions are known to haul out at the Snares Islands (48°S, 166°E) and there are only occasional reports of pupping presumed to have taken place on the main island (Crawley and Cameron, 1972, Cawthorn, 1993). Since 1998 however, albatross researchers on the island have surveyed the coastal areas where NZ sea lions have previously been known to frequent, confirming no further breeding at this site (Chilvers et al., 2007b).

1.3. Life history and annual cycle

Since not all breeding areas have been studied with the same effort or consistency over the decades, the vast majority of published research on the biology and life history of NZ sea lions has been obtained from the Sandy Bay colony on Enderby Island, Auckland Islands. As such, the following information may not necessarily reflect accurately what happens in other locations but is likely to be similar.

Female NZ sea lions reach sexual maturity around three years of age, but generally do not start breeding until between four to eight years old. The majority of the reproductively active female cohort is 7 to 23 years old, however the prime breeding period is thought to be 7 to 12 years of age (Childerhouse et al., 2010, Roberts et al., 2013). Males are thought to reach sexual maturity between five and nine years of age, but do not begin to hold harems until at least eight years old.

The seasonal cycles of breeding females and males are summarised in Figures 1.5a and 1.5b. Adult males arrive at Sandy Bay and establish territories along the beach in late November. Pregnant females congregate at nearby haul outs before arriving at the colony an average of two days before pupping to form harems of up to 25, attended by a dominant male (Chilvers et al., 2007a). Subordinate and bachelor bulls remain at the periphery, occasionally challenging the dominant bulls throughout the pupping and mating period. At the Sandy Bay colony there is extreme synchrony of births, with all pupping being concentrated within a one month period, and 69% of all births occurring within a two week period, one week either side of 26th December (Chilvers et al., 2007a). Females give birth to a single pup with males born significantly heavier than females, having mean birth masses of 10.8kg and 9.7kg respectively (Chilvers et al., 2007b).

Following parturition, mothers nurse their pups for an average of 8.6 days before leaving for their first foraging trip (Chilvers et al., 2007b). In contrast to phocids (true seals), otariids lactate for long periods during which they alternate between periods ashore attending their pup (on average 1.3 days) and foraging trips at sea (on average 2.2 days; Chilvers et al., 2006).

Figure 1.5 Summary of the annual cycle of breeding New Zealand sea lions (*Phocarctos hookeri*). A. female and B. male.

Within two weeks of parturition, females undergo postpartum oestrus and mating continues until mid-January, when mature males leave the colony and the harems disperse. In this time, there is increasing presence of younger, socially immature males, which maintain territories around groups of females but rarely are successful in mating (Cawthorn, 1993). From approximately the third week of January, females move their pups inland to the adjoining sward and dense scrub where they remain in small groups while their mothers are away on feeding excursions at sea (Augé et al., 2012a). Males roam widely at sea for the rest of the year, feeding alone or in small groups (Chilvers and Wilkinson, 2008). Females however are constrained to the vicinity of the Auckland Islands for the rest of the year, by the nutritional demands of their pup.

Otariids generally produce a high energy, high lipid milk to provide nutrition for pups to develop a thick blubber layer for thermoregulation as well as an energy reserve for the days of fasting while their mothers are at sea. The mean milk lipid concentration of NZ sea lions was found to be amongst the lowest reported for otariids, and was correlated positively with maternal body condition index (Riet-Sapriza et al., 2012). Protein concentrations were comparable to other otariid species, allowing for adequate postnatal growth. The lactation period lasts up to ten months (Childerhouse and Gales, 2001), with pups thought to be weaned in August or September.

Although not specifically described in NZ sea lions, embryonic diapause has been identified in at least thirteen species of phocids and seven otariids and is likely to also occur in NZ sea lions (Atkinson, 1997). This consists of obligate arrested development of the blastocyst after fertilisation and delayed implantation onto the wall of the uterus for between two and five months. Control of reactivation and implantation is unclear but may be related to decreasing photoperiod (Boyd, 1991). Embryonic diapause allows the time of birth and the time of mating to occur closely together, combining two potentially energetically expensive events, to reduce the overall cost (Boyd, 1991).

The average NZ sea lion reproductive rate (percentage of mature females giving birth each year) based on data from Sandy Bay has been estimated at 67% (Childerhouse et al., 2010). This is within

the range of other reports in otariids as summarised in Table 1.3. However, NZ sea lions have been identified as the lowest and slowest reproducing otariids with predicted average lifetime production of four pups; or when pup survival to eight weeks is taken into account, three pups (Chilvers et al., 2010). Modelling data in the same study estimated approximately 27% of females that survive to the age of three will never breed and 29% will not rear a pup that survives beyond eight weeks. The reason for this poor reproductive success is not known and warrants investigation. Factors that could contribute to poor reproductive success are discussed in section 1.4.

Table 1.3. Summary of reported otariid reproductive rates.

Species	Reproductive rate (age class if reported)	Reference
Fur seals		
Antarctic fur seal	71%	(Boyd, 1993)
Arctocephalus gazella	70-80% (5-11 years)	(Lunn et al., 1994)
Australian fur seal	53 ± 3% (3-20 years)	(Gibbens et al., 2010)
Arctocephalus pusillus doriferus		
New Zealand fur seal	67%	(Goldsworthy and Shaughnessy, 1994)
Arctocephalus forsteri	26-64%	(McKenzie et al., 2005)
Subantarctic fur seal	63% (8-13 years)	(Beauplet et al., 2006)
Arctocephalus tropicalis		
Sea lions		
Australian sea lion	71%	(Higgins and Gass, 1993)
Neophoca cinerea		
California sea lion	77% (4-16 years)	(Melin et al., 2012)
Zalophus californianus	56% (17-21 years)	
	80% (10-12 years)	(Hernández-Camacho et al., 2008a)
	73% (13-15 years)	
	61% (16-18 years)	
	52% (19-21 years)	
	6% (22-25 years)	
New Zealand sea lion	67% (3-28 years)	(Childerhouse et al., 2010)
Phocarctos hookeri	60-95% (8-13 years)	(Chilvers et al., 2010)
	40-70% (13-15 years)	
	5-10% (18-20 years)	
Steller sea lion	63%	(Pitcher and Calkins, 1981)
Eumetopias jubatus	69%	(Maniscalco et al., 2010)

1.4. Reproductive success

Reproductive success is defined here as the production of a viable offspring, including the phases of mating, gestation, and early pup survival to six weeks of age. An overview of the potential causes of failure at each stage is outlined in Figure 1.6. These may further be divided into non-infectious and infectious causes. Several of these factors impacting domestic species and pinnipeds have previously been summarised (Reijnders, 1984, Givens and Marley, 2008, Verstegen et al., 2008), however infectious causes will be elaborated here in line with the scope of this thesis.

1.4.1. ***Non-infectious causes of reproductive failure***

1.4.1.1. Host factors

Many aetiologies of reproductive failure including male and female infertility, hormonal abnormalities or primary anatomical defects are rarely reported in pinnipeds and have not been described in NZ sea lions, in part due to the impracticality of diagnosis in remote locations. Reproductive hormone analysis of adult NZ sea lion females has not been reported but could aid in determining early pregnancy rates and therefore the rate and stage of gestational failure. Due to a lack of suitably timed serum samples, hormonal analysis was not undertaken as part of this thesis and is not considered further.

Significant reproductive failure due to foetal mortality during mid to late gestation has been identified in Australian- (*Arctocephalus pusillus doriferus*) and NZ fur seals (*Arctocephalus forsteri*; McKenzie et al., 2005, Gibbens et al., 2010). This information for NZ sea lions would be indispensable to unravel the problem of low reproductive success, as due to dispersal and summer biased monitoring of major colonies, it is not clear whether abortion or premature parturition occur to a significant degree, as is seen routinely in more intensively monitored species. Similarly, inbreeding depression caused by genetic bottlenecks is well known to result in poor offspring fitness, infertility and reproductive failure (Amos et al., 2001). Microsatellite analysis has shown low genetic diversity

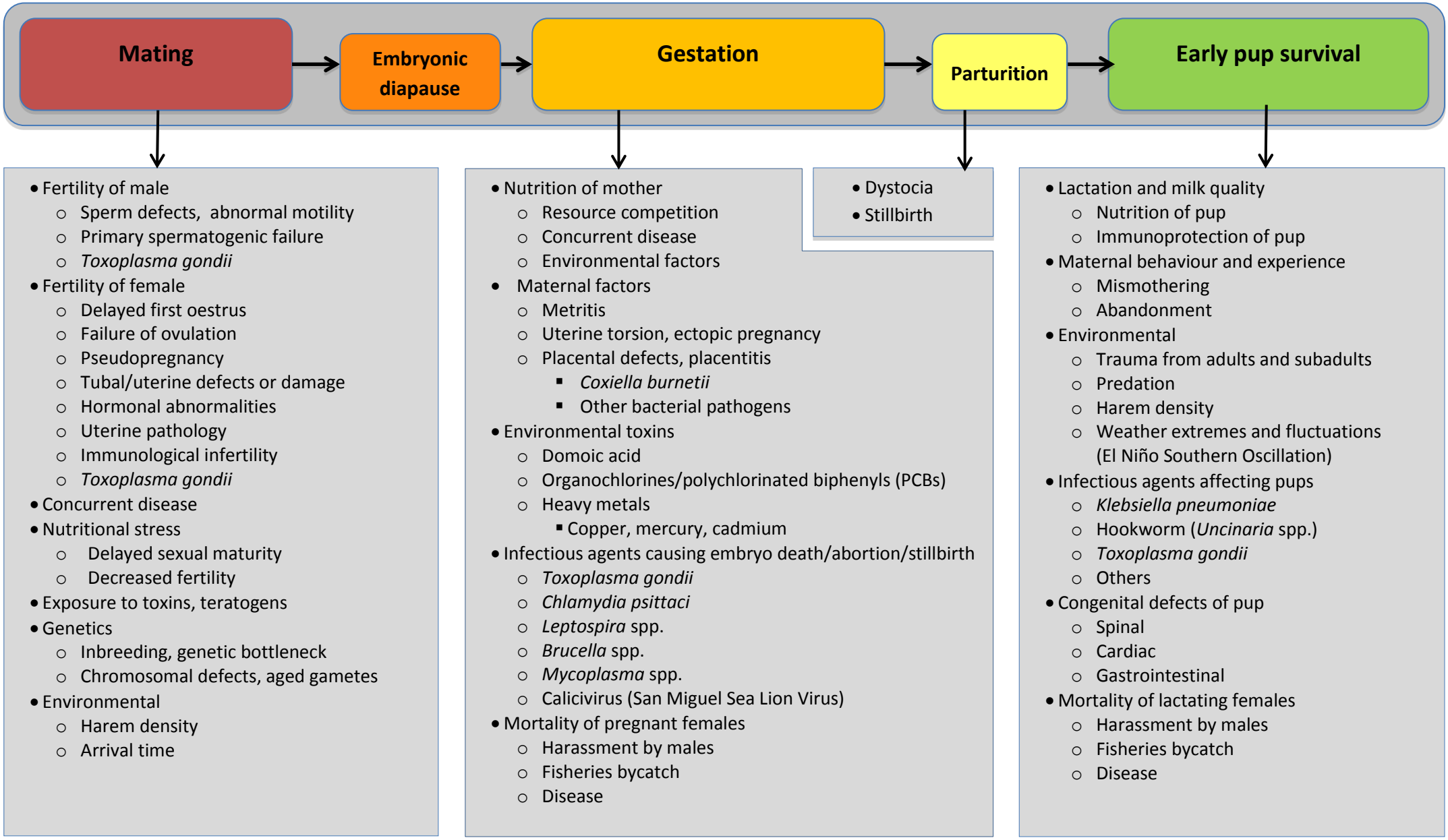

Figure 1.6. Summary of potential causes of poor reproductive success in New Zealand sea lions (*Phocarctos hookeri*).

in the NZ sea lion, which may be a contributing factor to ongoing poor reproductive success (Osborne, 2011).

Unlike in domestic dogs and cattle, where abnormal orientation of the foetus can be a contributing factor to dystocia with consequent stillbirth (Mee, 2008, Münnich and Küchenmeister, 2009), breech position of the foetus during parturition in Antarctic fur seals (*Arctocephalus gazella*) is significantly more common than cephalic presentation (1.5:1; Acevedo et al., 2008). Although pups born breech were delivered over a longer period of time, there were no associated adverse effects noted. Approximately 50% of NZ sea lion pups born at Sandy Bay are estimated to be delivered in breech orientation (BL Chilvers, unpublished data) but correlation with likelihood of stillbirth has not been investigated.

Reproductive failure has been reported in NZ sea lions in association with male harassment causing mortality of pregnant females and trauma to neonatal pups (Chilvers et al., 2005). However, the latter is predominantly density dependent and unlikely to be a major explanation due to comparatively low densities at the primary breeding sites of Sandy Bay and Dundas Island (Doidge et al., 1984, Reid and Forcada, 2005, Chilvers et al., 2007a). Elsewhere at more crowded otariid colonies such as Bird Island, South Georgia (Antarctic fur seals; Doidge et al., 1984) and La Mare aux Elephants, Amsterdam Island (subantarctic fur seals, *Arctocephalus tropicalis*; Georges and Guinet, 2000) however, incidental crushing and drowning of pups by adults is common, particularly when pups are young and poorly agile.

Maternal age does not appear to impact on pup mass, pupping date or pup mortality in NZ sea lions, however postpartum maternal mass was positively correlated with pup birth mass (Chilvers et al., 2007a). In other pinniped species, small and light primiparous females are more likely to pup later in the season, giving birth to smaller pups that are more likely to die of starvation (Doidge et al., 1984). Inexperienced females may also be less likely to wean offspring, hence nutritionally disadvantaging pups in the next season, as well as causing failure of a bond between the mother and the new pup

(Lunn et al., 1994). Alternatively in Steller sea lions (*Eumetopias jubatus*), extended maternal care by not weaning yearlings was shown to improve the survival rate of the juvenile to two and three years of age up to twofold (Maniscalco, 2014).

1.4.1.2. Environmental factors

Domoic acid is a neurotoxin produced by marine diatoms in the genus *Pseudo-nitzschia* that has been shown to cause premature parturition and adult mortality following ingestion by California sea lions (*Zalophus californianus*; Brodie et al., 2006). Affected adults displayed neurological symptoms such as ataxia, seizures and coma, corresponding to histological findings of neuronal atrophy and necrosis of the hippocampus and dentate gyrus (Silvagni et al., 2005). Toxins can cross the placenta of affected pregnant females and are thought to cause similar atrophy in the foetal brain, placental abruption and subsequent abortion (Brodie et al., 2006, Goldstein et al., 2009). *Pseudo-nitzschia* has been identified in New Zealand in shellfish (Rhodes et al., 1998) but no reports on marine mammal intoxication have been published.

Similarly, persistent organic pollutants such as organochlorines, particularly polychlorinated biphenyls (PCBs) and dichloro-diphenyl-trichloroethane (DDT) have been described to cause infertility and premature parturition in California sea lions (DeLong et al., 1973) and uterine stenosis in Baltic ringed seals (*Pusa hispida*; Helle et al., 1976). Consistent with the remoteness from industrial sites and sources of these pollutants, eight blubber samples from by-caught NZ sea lions around the Auckland Islands had PCB levels well below the suggested toxic thresholds for marine mammals (Donaldson, 2008).

Adverse local weather impacts on NZ sea lion pup survival are more severe at higher latitudes. While Sandy Bay has a south-facing aspect, it is sheltered from prevailing winds by Port Ross harbour. On the other hand, low lying Dundas Island and both Campbell Island colonies are exposed to wind, rain

and swells with minimal vegetation cover for pups to shelter (Maloney et al., 2012). Global fluctuations in climate and surface sea temperatures as a result of the El Niño Southern Oscillation has been strongly correlated with poor reproductive success in South American sea lions (*Otaria flavescens*; Soto et al., 2004). During a severe El Niño event in 1998, prey availability was extremely low resulting in approximately 1000 abortions and 100% mortality of those pups that were born alive (Soto et al., 2004). Environmental fluctuations have been hypothesised to have a greater negative impact on those species with a benthic foraging strategy, which is the case for approximately 50% of the lactating NZ sea lion females whose foraging specialisation has been studied at Sandy Bay (Costa, 2007, Chilvers and Wilkinson, 2009).

1.4.2. ***Infectious causes of reproductive failure***

1.4.2.1. Gestational failure

Various diseases have been associated with premature parturition, stillbirth and abortion in pinnipeds around the world. Stillborns form a small but consistent proportion of pup mortality at Sandy Bay, however they have never undergone investigation for signs of infectious disease.

Bacteria from the genus *Brucella* are well known to cause late gestation abortions and premature parturition in female domestic ruminants, as well as epididymitis and orchitis in males (Seleem et al., 2010). More recently, marine mammal strains of the zoonotic bacteria have been recognised, with infection reported to cause reproductive failure in cetaceans including placentitis in bottlenose dolphins (*Tursiops truncatus*; Miller et al., 1999) and epididymitis in a harbour porpoise (*Phocoena phocoena*; Foster et al., 2002). On the other hand, brucellae have been isolated from various apparently healthy pinnipeds, and where disease is evident, a respiratory affinity is most common, causing bronchopneumonia (Prenger-Berninghoff et al., 2008). The few reports of potential *Brucella*-associated reproductive failure in pinnipeds have not correlated pathologic findings with recovery of the pathogen (Goldstein et al., 2009, Lynch et al., 2011a). Serologic evidence of exposure has been

demonstrated around the world (Nymo et al., 2011). Although 57% seroprevalence was shown in adult female Australian fur seals (n=125) in south-eastern Australia, there were no positive sera detected in pre-weaned New Zealand fur seal pups (n=101) and adult female NZ sea lions from the Otago Peninsula (n=9) and only one weakly positive result from 138 samples from NZ sea lions on Enderby Island (Mackereth et al., 2005, Roe et al., 2010, Lynch et al., 2011a).

Leptospirosis has been identified as a prominent cause of periodic mass mortality and stranding in several pinniped species in waters off the western coast of North America, causing severe lymphoplasmacytic tubulointerstitial nephritis (Gulland et al., 1996, Colegrove et al., 2005). Additionally in this region, premature parturition in California sea lions and perinatal mortality or stillbirth in northern fur seal (*Callorhinus ursinus*) pups has been documented, associated with acute *Leptospira interrogans* serovar *pomona* infection (Gilmartin et al., 1976, Smith et al., 1977). Affected pups displayed sub-capsular haemorrhage of the liver and kidneys with free unclotted blood in the peritoneal cavity as well as hyphema. Where placentas were available for examination, focal necrotising placentitis was present, with leptospiral organisms demonstrated with Warthin Starry silver staining as well as isolated in culture. Seroprevalence amongst NZ pinnipeds appears to be low, despite the endemic presence of six serovars in domestic species on the NZ mainland (Roe et al., 2010). Transient suspicious or positive titres to serovars *pomona*, *hardjo* and *canicola* in thirteen NZ fur seal pups at the Otago Peninsula were found (Mackereth et al., 2005), however adult female NZ sea lions from the same location were negative (Roe et al., 2010).

Coxiella burnetii, a cosmopolitan bacterium that commonly causes abortions in domestic small ruminants is emerging as a potential cause of reproductive loss in pinnipeds. Thus far, *C. burnetii* has been documented in the placentas of Steller sea lions (Kersh et al., 2010, Kersh et al., 2012), Pacific harbour seals (*Phoca vitulina richardsi*; Lapointe et al., 1999, Kersh et al., 2012) and northern fur seals (Duncan et al., 2012) all on the Pacific coast of North America. In these cases there were typically focal or regionally extensive intracytoplasmic bacteria, markedly distending the

trophoblastic cells within the chorioallantoic villi, which was devoid of any associated inflammation. Despite such spectacular histologic findings, it is still to be demonstrated that placental infection would cause the death of the associated foetus. New Zealand is one of the few countries in the world that is thought to be free of *C. burnetii* (Anonymous, 2010), however no investigation has been reported about potential infection of NZ pinnipeds.

Another potential cause of reproductive loss with a worldwide distribution is the coccidian parasite *Toxoplasma gondii*. Unlike other coccidia, it is not host specific and can infect any warm blooded animal, including marine mammals and humans. Transplacental transmission of tachyzoites from infected pregnant women can lead to placentitis and abortion or congenital infection in the foetus, while immunocompromised patients develop fatal encephalitis or disseminated infection (Hill and Dubey, 2002). Experimental infection in rodents has also been shown to cause infertility in both males and females (Stahl et al., 1994, Terpsidis et al., 2009). Significant mortality associated with fatal toxoplasmosis has been identified in declining populations of southern sea otters (*Enhydra lutris nereis*; 16.2% of mortality; 17/105) in western USA (Kreuder et al., 2003) and Hector's dolphins (*Cephalorhynchus hectori*; 25%; 7/28) in NZ waters (Roe et al., 2013). Several reports of congenital infection in a southern sea otter foetus (Miller et al., 2008), a Risso's dolphin foetus (*Grampus griseus*; Resendes et al., 2002) and a California sea lion neonate (Ratcliffe and Worth, 1951) have described severe non-suppurative multi-systemic disease. Despite the fact that domestic and wild felids are the only known definitive hosts of the parasite able to shed infective oocysts in the faeces, serologic evidence of exposure has been documented in many pinniped species around the world, even in places that have never had resident cats, such as Antarctica (Jensen et al., 2012, Rengifo-Herrera et al., 2012). No seroprevalence data is currently available for NZ pinnipeds.

There are several potential pathogens that have not been definitively linked with reproductive failure in marine mammals, which should still be considered in an investigation. The role of *Mycoplasma* spp. as a contributor to abortions of Australian fur seals was proposed by Lynch et al.

(2011c). Although the organism was isolated from apparently healthy live animals and post mortem samples without any lesions, it was found with PCR (polymerase chain reaction) in 27% (3/11) of consistent inflammatory heart or lung lesions in aborted foetuses. It is likely that these organisms are part of the normal flora, but their involvement in reproductive loss is still unclear. *Chlamydia psittaci* is well known to cause placentitis, abortions and infertility in small ruminants, although the same clinical outcomes have not been described in marine mammals. Burek et al. (2005) showed that antibodies to *C. psittaci* are present in Steller sea lions, but their involvement in causing disease is not known. San Miguel sea lion virus, a calicivirus that is indistinguishable from vesicular exanthema of swine virus, was isolated originally from California sea lions with several syndromes of blistering skin, encephalitis, abortion, myocarditis and multiple haemorrhages (Smith et al., 1998). Disease outbreaks within the species were suggested to be enzootic, and although seroprevalence has been documented in other northern hemisphere pinniped species, there have been no other accounts of disease.

1.4.2.2. Early pup survival

Once a pup has successfully been born, the primary infectious cause of neonatal death in NZ sea lions at Sandy Bay is infection with the bacterium *Klebsiella pneumoniae*. As alluded to previously, this agent caused mass pup mortality in the 2001/02 and 2002/03 seasons and recent work has shown that this disease may have become enzootic in the population, with large proportions of annual pup mortality due to *K. pneumoniae* disease including septicaemia with meningitis and polyarthritis (WD Roe, unpublished data). Almost all adult females tested were shown to be seropositive for antibodies to *Klebsiella* spp., while only 16% of pups demonstrated detectable serum IgG antibody, interpreted as a failure of passive immunity transfer (Castinel et al., 2008). Research into pathogenesis and reservoirs of infection is ongoing.

Intestinal infestation with the hookworm (*Uncinaria* spp.) parasite is common in young pups at Sandy Bay, with infection occurring through transfer of infective third stage larvae (L3) via the transmammary route in peripartum colostrum. There is much variation in the clinical appearance and neonatal mortality rate associated with hookworm infection in otariids worldwide which may be underpinned by local environmental factors including substrate type and host population density as summarised in Table 1.4. In some species, including Juan Fernandez fur seals (*Arctocephalus philippii*; Sepúlveda, 1998), Steller sea lions (Lyons et al., 2003) and northern fur seals at St Paul Island, Alaska (Ionita et al., 2008, Lyons et al., 2012); pups have low hookworm intensity and no gross lesions indicative of enteritis or anaemia. These colonies share the conditions of predominantly rocky substrate and low population density, both of which are unfavourable for hookworm survival and transmission. South American sea lions present an intermediate case of moderate hookworm intensity and sandy substrate but hookworm infection is considered incidental due to lack of gross lesions in the intestinal tract (Berón-Vera et al., 2004). On the other hand, California sea lions and northern fur seals in high density, sandy colonies on San Miguel Island, California are reported to be subject to high intensity burdens and high levels of hookworm-associated neonatal mortality (Lyons et al., 2001).The typical findings of enteritis here are also compounded by unusual complications of aberrant peritoneal migration of hookworms and an association with septicaemia (Spraker et al., 2004, Spraker et al., 2007). The sandy substrate of these pinniped colonies is thought to be favourable for hookworm L3 persistence and this is consistent with higher intensities seen in species living there compared to rocky locations. An exception is seen in Australian sea lions (*Neophoca cinerea*) where very high hookworm intensity is seen at both sandy and rocky colonies, but the reason for this is unclear. NZ sea lions have been shown to have high prevalence and intensity of hookworm infestation and although gross pathology of haemorrhagic enteritis is commonly seen, it is the primary cause of death in a relatively low number of pups (Castinel et al., 2007a). There may however be a local effect at Sandy Bay as the beach sand washes

Table 1.4 Summary of reported hookworm findings in otariid species.

Species	Location	Hookworm infection intensity*	Hookworm prevalence	Hookworm-associated gross pathology	Predominant substrate type	Otariid population density	Reference
Steller sea lion *Eumetopias jubatus*	Rogue Reef, Oregon, USA	-	0% of 50 live and 6 dead pups	None	Rock	Increasing	(Lyons et al., 2003)
Juan Fernandez fur seal *Arctocephalus philippii*	Alejandro Selkirk Island, Chile	Low 1-151 worms/pup, mean 17	60% of 60 dead pups	None	Rock	Low, increasing	(Sepúlveda, 1998)
Northern fur seal *Callorhinus ursinus*	St Paul Island, Alaska, USA	Low 1-408 worms/pup (3/4 pups had ≤2 worms)	6.25% of 64 dead pups	None	Rock	Moderate, decreasing	(Ionita et al., 2008, Lyons et al., 2012)
South American sea lion *Otaria flavescens*	Punta León, Argentina	Medium 1-451 worms/pup, mean female 93, male 230	50% of 31 dead pups	None	Sand	Low, increasing	(Berón-Vera et al., 2004, Dans et al., 2004)
California sea lion *Zalophus californianus*	San Miguel Island, California, USA	High 20-2634 worms/pup, mean 612 (2000) 39-2766 worms/pup, mean 575 (2003)	100% of 25 live and 204 dead pups	Enteritis/bacteremia a major diagnosis in 145/204 pups, peritoneal migration in 13/104 pups	Sand	High, increasing	(Lyons et al., 2001, Lyons et al., 2005, Lyons et al., 2011a, Spraker et al., 2007)
Northern fur seal *Callorhinus ursinus*	San Miguel Island, California, USA	High 4-2142 worms/pup, mean 760 (2000) 3-2,344 worms/pup, mean 695 (2003)	95% of 20 live pups	Enteritis/bacteremia common. Peritoneal migration in 35/57 pups	Sand	High, increasing	(DeLong et al., 2009, Lyons et al., 2001, Lyons et al., 2011a)
New Zealand sea lion *Phocarctos hookeri*	Sandy Bay, Enderby Island, NZ	High 136-3323 worms/pup, mean 824	75% of 200 live pups	Enteritis a contributor to mortality in 60/455 pups	Sand	Low, decreasing	(Castinel et al., 2007a, Castinel et al., 2007b, Chilvers et al., 2009)
Australian sea lion *Neophoca cinerea*	Dangerous Reef and Seal Bay, SA, Australia	Very High 1-8880 worms/pup, mean 2138±552	100% of 58 live pups	Not described	Rock/Sand	High	(Marcus et al., 2014)

*Note mean hookworm value is given for male and female pups separately if significantly different.

away every winter exposing underlying pebbles, hence naturally decreasing the infective larval burden and likely impeding the survival of those that remain (Chilvers et al., 2009). Additionally, adults and pups migrate off the beach onto the grassy sward within a month of the median pupping date, reducing percutaneous transmission from sand.

In addition to external factors, genetic predisposition has also been implicated in the propensity for NZ sea lion pups to succumb to hookworm infection, regardless of burden intensity. The link identified between inbreeding and hookworm–associated mortality in California sea lion pups was not seen in NZ sea lions, further there was relatively high microsatellite allelic diversity amongst the Sandy Bay population (Acevedo-Whitehouse et al., 2006, Acevedo-Whitehouse et al., 2009). On the other hand, homozygosity at a particular microsatellite locus was associated with a strong predisposition to anaemia in both species, with a certain genotype being associated with significantly lower platelet counts (Acevedo-Whitehouse et al., 2009). Such pups would benefit from early clearing of intestinal parasites and prevention of worm attachment but selection in favour of pups with these anaemia-susceptible genotypes would likely result in the need to perpetuate provision of anthelmintics. Anthelmintic treatment has been attempted in wild NZ sea lion and northern fur seal pups with successful reduction of worm burdens, improved growth rates and early survival (Chilvers et al., 2009, DeLong et al., 2009).

1.5. Thesis outline and aims

This research presented in this thesis aims to investigate potential infectious causes for the described poor reproductive success of the NZ sea lion. Specific objectives that were addressed included:

1. Quantify stillbirth as a cause of NZ sea lion reproductive failure at Sandy Bay, Enderby Island and identify possible impacts of infectious disease from archived necropsy records and tissues
2. Determine the level of exposure to *Toxoplasma gondii* amongst NZ sea lions from Sandy Bay, Enderby Island compared to Otago Peninsula and Stewart Island and identify potential impacts of the pathogen on reproductive success
3. Investigate long term survival and reproductive success of NZ sea lions that were treated with an anthelmintic as pups to remove hookworm burden and compare to a control group

These objectives were accomplished in three data chapters presented in this thesis. Chapter Two investigates histopathology of archived tissues from 37 stillborn pups born at Sandy Bay, Enderby Island, to diagnose cause of death and determine whether inflammatory or infectious processes contributed to these deaths. Chapter Three examines archived sera from adult NZ sea lions for antibody presence, indicating exposure to the pathogen *T. gondii*. Seroprevalence is compared between animals from mainland sites (Otago Peninsula and Stewart Island) and sub-Antarctic Enderby Island. Chapter Four investigates the longer term impacts of neonatal hookworm infection of NZ sea lion pups on survival and reproduction using survival analysis.

The three data chapters of this thesis have been written in a format to facilitate journal publication (Chapter Two, *The Veterinary Journal*; Chapter Three, *Veterinary Parasitology*; Chapter Four, *International Journal of Parasitology*) and consequently there is some unavoidable repetition and overlap between the literature review and the chapters. Additional methodology and further

information not included in the submitted manuscripts are presented as appendices. All references for literature cited are presented at the end of the thesis.

Chapter Two.

Pathology of stillborn New Zealand sea lion pups from Enderby Island, Auckland Islands, 1998-2012

2.1. Abstract

Stillbirth is a small and often cryptic fraction of neonatal mortality in mammals including pinnipeds. Often, it is difficult to assign an aetiological diagnosis to these cases at gross necropsy due to the interlinked relationship between maternal, foetal and placental factors. As part of an investigation into the poor reproductive success of the threatened New Zealand sea lion (*Phocarctos hookeri*), archived tissues from 37 stillborn pups born on Enderby Island between 1998 and 2012 were examined using histopathological techniques to identify potential infectious cause of death. Since placentas have not been examined historically, ten placentas were collected opportunistically in 2012 and examined for signs of disease. No infectious aetiologies were identified in stillborn pups or placentas. Four stillborn pups had evidence of pneumonia with neutrophilic or histiocytic infiltration, however 86% of all pups examined had aspirated squames in the respiratory tract, without meconium. It is unclear whether these pups experienced foetal distress during parturition or this finding is normal for the species. This study forms an important baseline for further examination of stillborn pups and placentas in the future, as pup mortality is investigated as a contributor to the species' decline.

2.1.1. ***Keywords***

Foetal distress, pinniped, placenta, pneumonia, squames, stillborn.

2.2. Introduction

Stillbirth is an uncommon but consistent cause of reproductive failure in mammals, including humans. While the exact aetiology is often unknown, it is clear that there is a complex interplay between foetal, maternal and placental factors, of which all must harmonise to produce a viable offspring. In domestic animal species and even more so in wild populations, there is rarely any pathological investigation of stillborn foetuses, perhaps due to the low yield of a definitive diagnosis, even following extensive diagnostic investigation (Schlafer, 2008). Stillbirth, abortion and premature parturition have been regularly reported in routinely monitored pinniped species (Goldstein et al., 2009, Lynch et al., 2011a), but in remote sub-Antarctic breeding sites such as those utilised by the threatened New Zealand (NZ) sea lion (*Phocarctos hookeri*) little is known about the occurrence or extent of late gestational failure.

NZ sea lions are the only pinniped endemic to New Zealand, with over 99% of breeding restricted to the NZ sub-Antarctic islands between 50-53°S. The Sandy Bay colony at Enderby Island (50°30'S, 166°17'E) in the Auckland Islands archipelago is the species' second largest breeding site and the most intensively studied. In addition to restricted distribution, pup production is in decline, with almost half as many pups born in the most recent season (2013/14), compared to 16 years earlier when pup production was at an apparent peak (Childerhouse et al., 2014). Reproductive rate and average lifetime pup production estimates from Sandy Bay have been reported to be low, with no clear reason identified for such poor reproductive success (Childerhouse et al., 2010, Chilvers et al., 2010). Although this site is the most well studied, outside of the austral summer field season (mid-December to mid-February) the island is uninhabited and any early reproductive losses would be unnoticed.

Reported stillbirth rates (proportion of stillbirths from total pup mortality) in otariids are generally low including 7% in Antarctic fur seals (*Arctocephalus gazella*; Reid and Forcada, 2005), 7.6% in Australian sea lions (*Neophoca cinerea*; McIntosh and Kennedy, 2013), 9% in northern fur seals

(*Callorhinus ursinus*; Spraker and Lander, 2010), 2.6% in South American fur seals (*Arctocephalus australis gracilis*; Seguel et al., 2013) and 2.3% in South American sea lions (*Otaria flavescens*; Soto et al., 2004). The known stillbirth rate for NZ sea lions at Sandy Bay is 4.2% but this should be recognised as a minimum due to lack of quantification of early season abortions and premature parturitions (Castinel et al., 2007b).

Infectious disease is well known to impact on population dynamics at Sandy Bay, with three known bacterial epizootics contributing to mass pup mortality of between 21-53% of pup production in the monitored portion (December to February) of affected seasons (Baker, 1999, Wilkinson et al., 2004). In relation to late gestational failure including abortion and stillbirth, several infectious agents have been investigated in pinnipeds including *Brucella* spp., *Leptospira* spp., *Coxiella burnetii*, *Chlamydia psittaci*, *Mycoplasma* spp., *Toxoplasma gondii* and calicivirus (Burek et al., 2003, Lynch et al., 2011a, Lynch et al., 2011c, Minor et al., 2013). While a causal link has not been established for the role of many of these pathogens in pinniped reproductive failure and many non-infectious factors may be involved, it is imperative to investigate the contributing factors in NZ sea lions.

Whilst pup necropsies have been undertaken routinely at Sandy Bay since 1998, and most cases have corresponding archived formalinised tissues, little further investigation has been performed. Similarly, there has been no examination or preservation of placental tissue, thus an important component of late gestational failure was excluded from analysis. Accordingly, the aim of this study was to retrospectively evaluate the stillborn cohort of NZ sea lion neonatal mortality between 1998 and 2012 for evidence of infectious aetiology. Additionally, ten placentas collected on a single day in December 2012 were also available for analysis to determine potential infectious pathology.

2.3. Materials and Methods

Between 1998 and 2012, from mid-December to mid-February each year, all dead pups were collected at least once daily from the Sandy Bay colony, on Enderby Island, Auckland Islands (50°30'S, 166°17'E). The dead pups were identified by sex and flipper tag if present, and morphometric data was collected from 2001 onwards including weight, body length (from nose to tail), axillary girth and sternal blubber depth. A full necropsy was then undertaken as summarised by Castinel et al. (2007b) by veterinarians or trained researchers. Tissue samples including lung, heart, liver, spleen, kidney, ovaries or testes, stomach, intestine, pancreas, adrenal gland, thyroid, tongue, skeletal muscle, diaphragm, trachea, lymph nodes, cerebrum and cerebellum were preserved in 10% neutral buffered formalin for histopathology. Samples of lesions were frozen in liquid nitrogen for microbiology with transport to and further processing at Massey University, Palmerston North, New Zealand.

The depth of investigation and precision of necropsy records varied from year to year. NZ sea lion necropsy records were reviewed and cases of stillbirth were identified by records of either visual confirmation of delivery of a dead pup or consistent necropsy findings including pulmonary atelectasis, fresh umbilicus, meconium in the colon and carcass decomposition such as sloughing of the hair coat and tissue autolysis (Spraker and Lander, 2010). Where formalin fixed tissues were available, they were trimmed, embedded in paraffin, sectioned at 4µm and stained with haematoxylin and eosin for histopathologic examination with grading of common findings on a scale of 0 to 3 (not present to severe) as outlined in Appendix 1. Special stains were used when required such as Gram stain for detection of bacteria and Periodic Acid Schiff (PAS) for identification of meconium.

Fresh placentas were collected as soon as possible after expulsion at Sandy Bay on 22nd December 2012. Sections were sampled from the chorioallantoic membranes and zonary placenta as well as

any regions that appeared abnormal grossly. These were preserved and processed as described for pup tissues.

The student's T test with two tails was used to compare morphometric parameters between live- and stillborn pups and differences were considered significant when $P<0.05$.

2.4. Results

Between 1998/99 and 2011/12, 812 pups had been necropsied at Sandy Bay, Enderby Island. Forty nine pups (26 male, 22 female, 1 unknown; Figure 2.1) were identified as stillborn, of which 37 (20 male and 17 female) had archived samples in formalin that were processed for histopathology. The median date of birth for stillborn pups was 20th December (n=49; range 5th December-30th January). This is approximately a week prior to the median birth date for live born pups (26-27 December; Chilvers et al., 2007a).

In 2011/12, there were no necropsies carried out in the first half of the season. Post mortems were undertaken from 10th January onwards in that year, well after the median date of birth for stillborns. Therefore, stillbirth data from this season should be treated with caution. When data from only entire seasons are included (1998/99 to 2010/11) this corresponds to a stillbirth rate of 6%. Annual stillbirth rates over this period ranged from 1.7% in 2002/03 to 28% in 2010/11 (Figure 2.2).

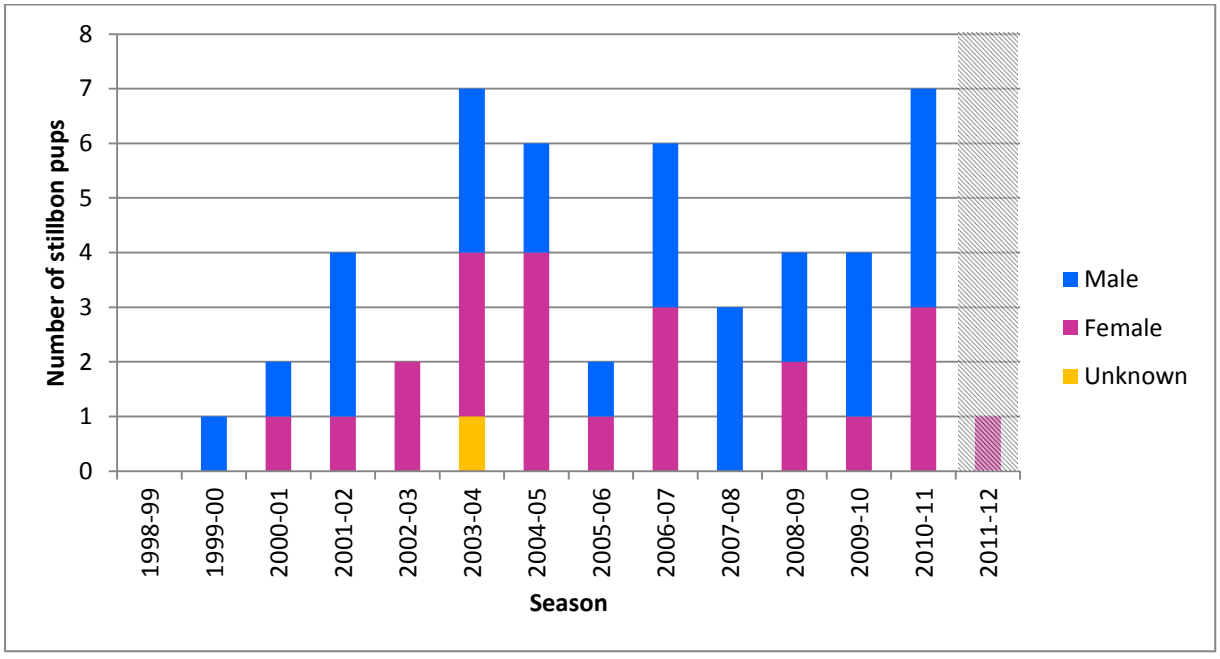

Figure 2.1. Total recorded stillborn New Zealand sea lion (*Phocarctos hookeri*) pups necropsied at Sandy Bay, Enderby Island.Note necropsies were only undertaken after 10th January in 2011-12 (hatched area).

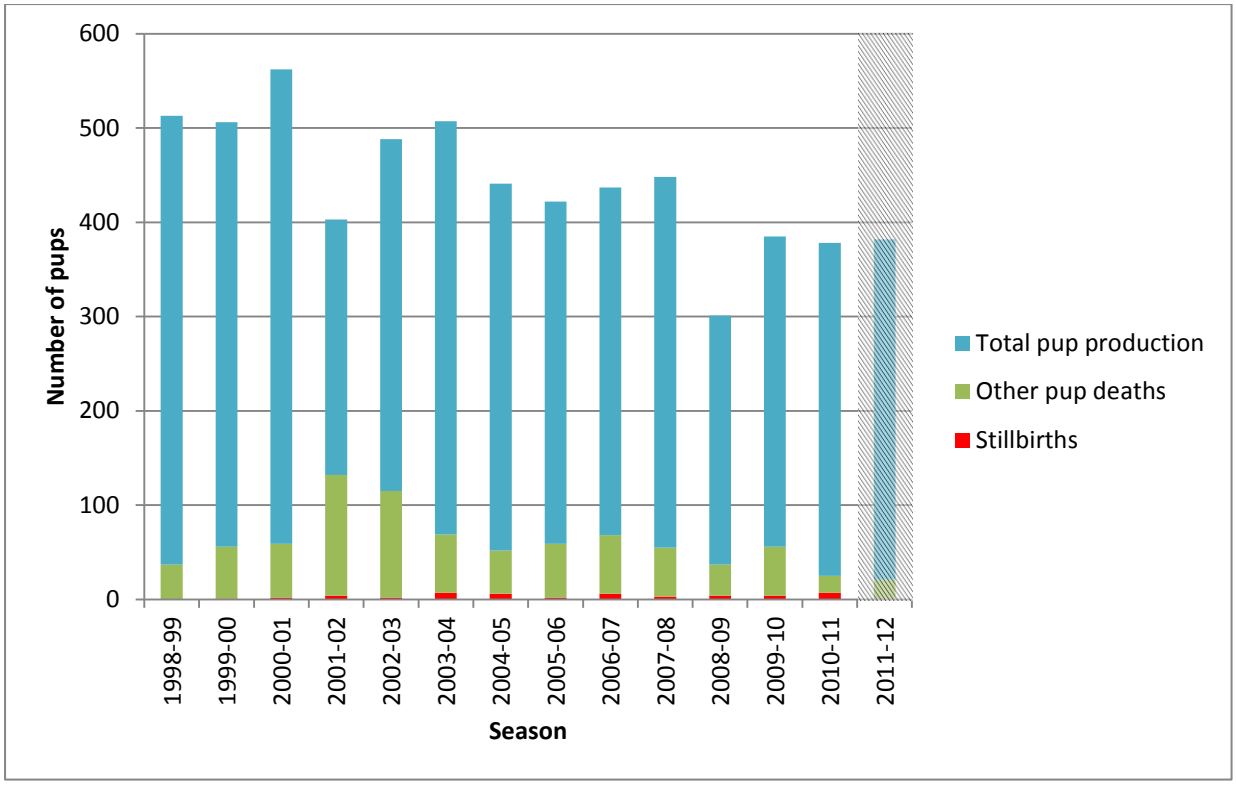

Figure 2.2 Comparison of stillborn New Zealand sea lions (*Phocarctos hookeri*) to total pups necropsied and total pup production at Sandy Bay, Enderby Island. Note incomplete necropsy schedule in 2011-12 (hatched area).

Morphometric data was available for 33 stillborn pups as outlined in Table 2.1. The general trend was that stillborns were lighter and longer, with a smaller axillary girth than live born pups. This was significant in males for length (t=-3.582, P=0.001) and girth (t=2.122, P=0.037) and in females for mass (t=2.265, P=0.026) and girth (t=2.260, P=0.027). Average sternal blubber depth was 9.6mm and 9.4mm for male and female stillborn pups respectively.

Table 2.1. Comparison of morphometric parameters of a sample of live born and stillborn New Zealand sea lion (*Phocarctos hookeri*) pups at Sandy Bay, Enderby Island (mean ± standard deviation).

	Male live born (n=58)	Male stillborn (n=17)	Female live born (n=60)	Female stillborn (n=16)
Mass (kg)	10.6 ± 1.4	10.2 ± 1.6	9.7 ± 0.9*	8.8 ± 1.52*
Length (cm)	77.6 ± 0.35**	81.4 ± 4.37**	74.8 ± 0.32	77 ± 5.65
Axillary girth (cm)	46.3 ± 0.26*	44.6 ± 3.3*	45.0 ± 0.31*	43.0 ± 3.54*
Blubber depth (mm)		9.6 ± 3.59		9.4 ± 3.05
Reference	(Chilvers et al., 2007a)		(Chilvers et al., 2007a)	

* indicates significantly different (P<0.05)
** indicates significantly different (P<0.01)

Considering the circumstances of collection in the field, existing decomposition of tissues at necropsy and the length of storage of some of the samples, the level of preservation was generally good. Tissues from four animals (11%) were severely autolysed, making it difficult to identify cell types and therefore accurately draw conclusions from histopathology. One of these was the only sample on which evidence of post mortem bacterial invasion (with a Gram positive rod) was evident on microscopic examination.

2.4.1. ***Stillborn pups***

Common histopathologic findings from stillborn pups are summarised in Table 2.2 and full results are presented in Appendix 2. Lung sections varied from completely atelectic to mostly collapsed with slight peripheral aeration. In the majority of pups examined, there was some degree of foreign material present in the small airways including homogenous eosinophilic material (likely amniotic fluid), sloughed squamous epithelial cells, cellular debris, alveolar macrophages and inflammatory cells.

Table 2.2. Summary of histopathologic findings and severity in stillborn New Zealand sea lion (*Phocarctos hookeri*) pups.

Histopathology finding	Total	Mild	Moderate	Marked
Lung				
Atelectasis	36/36 (100%)	2	17	17
Eosinophilic fluid in airways	26/36 (72%)	9	12	5
Squames in airways	31/36 (86%)	11	15	5
Infiltrates into airways:				
Alveolar macrophages	18/32 (56%)	10	7	1
Multinucleated giant cells	2/32 (6%)	1	1	
Neutrophils	4/32 (13%)	4		
Meconium in airways	0/32 (0%)			
Liver				
Hepatocyte glycogen vacuolation	27/31 (87%)	9	9	9

In four animals (13%) there was moderate to severe infiltration of alveolar macrophages with neutrophils into the alveolar spaces, in one case with fibrin admixed with proteinaceous fluid in the

interstitium (Figure 2.4a) and in the other three cases in association with squame aspiration (Figures 2.4b-d). Multinucleated giant cell infiltration was identified in two of these cases (Figure 2.4d). Aspirated squames were not always associated with inflammation. In cases with squame aspiration that were not severely decomposed, 59% (16/27) were associated with alveolar macrophages and with lesion grading there appeared to be a positive correlation between degree of aspiration and intensity of histiocytic response. Only one of the animals that did not have squames present (20%; 1/5) had evidence of alveolar macrophage infiltration and in this case was mild. There was variation in the distribution of squames when present, usually appearing diffusely throughout the airways, in conjunction with amniotic fluid, cellular debris and varying levels of inflammatory response. In some cases however, the accumulation of squames was multifocally severe (Figures 2.4b,c), completely filling an entire airway, while other areas were not affected at all. In these dense squame accretions there was no associated inflammatory response. Small round pink-orange globules suspicious of meconium were seen occasionally within airways but were not confirmed on PAS staining.

Marked diffuse vacuolation of the liver due to storage of glycogen was exhibited in 87% (27/31) of pups, in most cases obscuring the lobular structure on low power (Figure 2.5a). Hepatic extra-medullary haematopoiesis was common, both surrounding portal tracts and sinusoids (Figure 2.5b). In four animals however, there was markedly reduced vacuolation, rather resembling the histologic structure of an adult liver (Figures 2.5c,d), which was interpreted as glycogen depletion. One of these animals had severely decomposed tissues with post mortem bacterial invasion, which may have contributed to glycogen breakdown in that case.

All other tissues examined did not exhibit any signs of inflammation, disease agents or abnormalities.

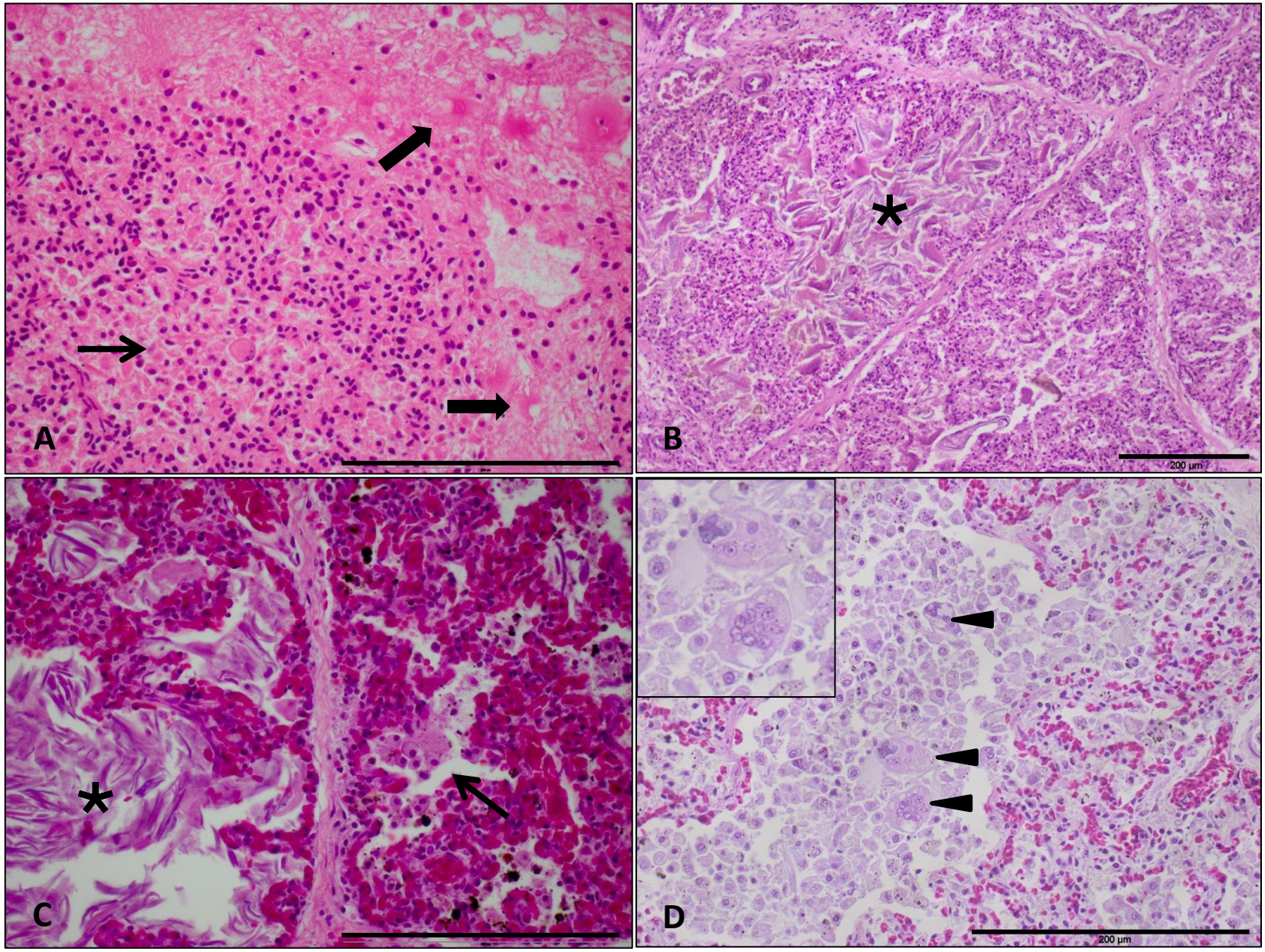

Figure 2.3 Histopathology lesions of stillborn New Zealand sea lion (*Phocarctos hookeri*) lungs. Haematoxylin and eosin. Scale bar denotes 200µm. A. Severe fibrinous neutrophilic interstitial pneumonia; heavy arrows denote fibrin and light arrow denotes alveolar infiltrate; B. Severe aspiration of squames (asterisk) without inflammatory response; C. Lobar distribution of squame aspiration (asterisk) and histocytic response (arrow); D. Severe histocytic and multinucleated giant cell (arrowheads and inset) response in association with aspirated squames.

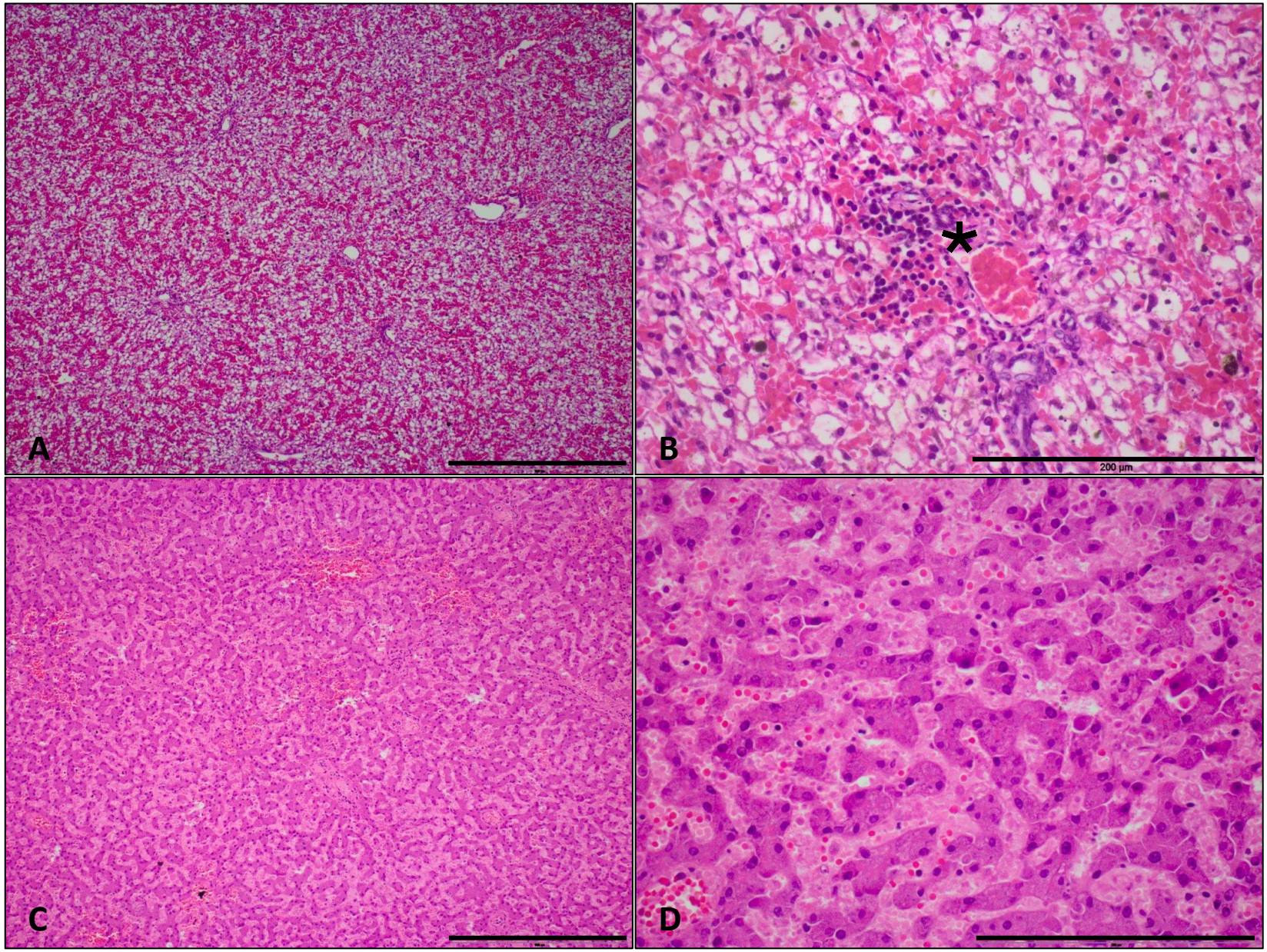

Figure 2.4 Histopathology of stillborn New Zealand sea lion (*Phocarctos hookeri*) livers. Haematoxylin and eosin. A. Diffuse glycogen vacuolation of hepatocytes, scale bar = 500µm; B. Glycogen vacuolation of hepatocytes with periportal extramedullary haematopoiesis (asterisk), scale bar = 200µm; C. Hepatic parenchyma without vacuolation, scale bar = 500µm; D. Hepatic parenchyma without vacuolation, scale bar = 200µm.

2.4.2. ***Placentas***

Ten fresh placentas were available for retrieval on 22nd December 2012 at Sandy Bay, Enderby Island. Pups associated with these placentas were observed alive and apparently healthy from distance examination. No gross abnormalities were noted on external examination of placentas (Appendix 3).

On histological examination, there was no evidence of inflammation or abnormality in the placental tissue or associated chorioallantoic membranes. The zonary placentas contained a haemophagous zone with associated marginal haematoma, adjacent to a large zone of chorionic villi (Appendix 4).

2.5. Discussion

Investigation of stillbirth beyond gross examination is often overlooked to the detriment of a comprehensive survey of neonatal mortality. This study has begun to fill this knowledge gap in NZ sea lions by investigating archived tissue samples of stillborn pups collected over a 14 year period and a small pilot sample of apparently normal placentas.

A consistent finding in 86% (31/36) of stillborn NZ sea lion pups examined histologically was the presence of sloughed squamous epithelial cells within the small airways and alveoli of the lungs, often with alveolar macrophages present. In four cases there was associated neutrophil infiltration (aspiration pneumonia). Squames are thought to build up in amniotic fluid throughout gestation by exfoliation of foetal skin and to a lesser extent the oropharyngeal mucosa. These findings are not uncommon in the human and domestic animal literature but the pathophysiology of periparturient foetal aspiration has not been fully established (Lopez and Bildfell, 1992, Alonso-Spilsbury et al., 2005). Large volumes of squamous cells within the lungs are commonly associated with foetal respiratory distress and anoxia *in utero*, following aspiration of amniotic fluid, and often meconium (Hoffman Jr et al., 1974, Gill, 2001). However, there are conflicting hypotheses whether any amniotic fluid present in the lungs should be considered abnormal (Cruickshank, 1949). Keratin within the airways is well known to invoke a dose dependent histiocytic inflammatory response, transiently recruiting alveolar macrophages and low numbers of neutrophils to the alveoli but in experimental studies on neonatal rats, meconium invoked a significantly more potent acute inflammatory response (Martínez-Burnes et al., 2001, Martínez-Burnes et al., 2002). To our knowledge, there are no published reports of meconium aspiration in foetal pinnipeds, consistent with our findings in NZ sea lion pups. On the other hand, the periparturient aspiration of squamous epithelial cells has been noted sporadically amongst the pinniped pathology literature. Moderate to severe aspiration of squames and other

debris was identified in 56% (30/59) of premature California sea lion (*Zalophus californianus*) pups examined histologically (Goldstein et al., 2009). In some of these cases it was suggested to be severe enough to compromise initial expansion of the lungs, however an associated inflammatory response was not reported. Lynch and colleagues (2011a) also found a high prevalence (86%; 25/29) of squamous cells in the lungs of aborted Australian fur seal (*Arctocephalus pusillus doriferus*) pups, of which six had concurrent lymphoplasmacytic pneumonia. Of the stillborn northern fur seals examined histologically by Spraker et al. (2010), only 2 pups (3%; 2/63) were identified to have pneumonia (one of these with concurrent placentitis), however squame aspiration was not reported. Although all four NZ sea lion pups with diagnosed acute pulmonary inflammation had squames in their airways, the majority with squame aspiration had little to no inflammatory response, so its significance is unclear. Without the finding of meconium aspiration in these animals, it is not possible to determine whether these pups aspirated amniotic fluid during a period of periparturient respiratory distress, as has been claimed for squame aspiration in some studies (Lopez and Bildfell, 1992, Martínez-Burnes et al., 2002).

Dystocia was recognised as a common cause of stillbirth and perinatal mortality in northern fur seals, especially in small females giving birth to large pups (Spraker and Lander, 2010) and could give rise to respiratory distress of the foetus during parturition. Although morphometric data is not available for pregnant females, it is likely that dystocia also occurs in NZ sea lions, as some particularly large pups (up to 13.2kg) were amongst the stillborn cohort. These would be consistent with foetal-pelvic disproportion described in domestic cattle, which is more common in male offspring, with high calf birth weight often being a more important factor than small maternal pelvic size (Meijering, 1984, Mee, 2008). Abnormal presentation of the foetus at delivery is also a contributing factor to dystocia in domestic animals but in Antarctic fur seals at least, breech presentation was more common than cephalic and although the former was longer in duration, it was not reported to be

associated with foetal death (Acevedo et al., 2008). Foetal orientation at parturition does not appear to be a risk factor for dystocia in NZ sea lions with approximately 50% of pups born in breech position but correlation with risk of stillbirth has not been assessed (BL Chilvers, unpublished data).

Normal neonatal hepatocytes contain autophagosomes filled with glycogen, visible microscopically as distended 'water clear' cytoplasm (Phillips et al., 1967), as seen in the vast majority of NZ stillborn pups. This glycogen serves the newborn with a system of glucose homeostasis to supply energy to the brain, heart and organs in the immediate postnatal period when the maternal nutrient supply is cut off and suckling has not yet begun. Glycogen autophagy is upregulated under the influence of glucagon immediately after birth and maintained at high levels for around 12 hours, after which time the vacuolated microscopic structure of the hepatocytes has all but disappeared (Phillips et al., 1967). This mechanism is important to prevent hypoglycaemia or catabolism of foetal tissue proteins for energy, while alternative production routes for glucose, such as gluconeogenesis are not yet fully developed (Kotoulas et al., 2006). It has been well described that humans and mice that are deficient in hepatic glycogen at birth, have poorer chances of survival and often die due to the effects of hypoglycaemia and energy depletion (Kuma et al., 2004). Four (11%) of the examined stillborn NZ sea lion pups had no evidence of hepatic glycogen vacuolation on histopathology, a finding not previously reported in the pinniped pathology literature. One of these pups was severely decomposed and one did not have recorded morphometric data but the remaining two were born well below the mean stillborn weights for their sex, with scant to minimal (1-4mm) blubber coverage, the least fat stores of all pups examined. These pups are consistent with human babies that have undergone intrauterine growth restriction, often delivered small for gestational age, with minimal subcutaneous fat, small lungs, liver and thymus, and low liver glycogen levels (Wigglesworth, 1967, Brodsky and Christou, 2004). This syndrome in humans has been suggested as a consequence of foetal under-nutrition *in*

utero, by either inadequate nutrition of the mother or of impairment of maternal-foetal transfer of nutrients due to placental pathology or foetal factors such as genetic abnormalities (Brodsky and Christou, 2004). Further research including examination of corresponding placentas as well as evaluating maternal body condition is required to better assess this link in NZ sea lions.

The low prevalence of inflammatory conditions in the NZ sea lion stillborn pups is in contrast to other otariid studies that have found relatively high prevalence on both gross and histopathologic examination. Spraker and Lander (2010) undertook the most extensive study of mortality in a pinniped species to date, with full necropsies of over 3000 northern fur seals of all age classes over twenty years. Amongst the stillbirth fraction, gross pathological lesions were identified in 76% (191/252) of animals, but only 27% of the pups examined histologically (17/63) had inflammatory lesions. The most common finding was placentitis (14/17), with pneumonia, enteritis, pulmonary oedema or mild hepatitis occurring only in single cases. In premature California sea lion pups from San Miguel Island, inflammatory lesions were also present in the majority of cases (50/59) (Goldstein et al., 2009). Although nine of these pups were concurrently suffering domoic acid toxicity, bacterial infections (31/59) affecting pup organs or the placenta and inflammation of unknown aetiology (26/59) predominantly in the lung and brain were well represented. Lynch and colleagues (2011a) identified inflammatory infiltrates in primarily lungs (8/29) and hearts (8/30) of several aborted Australian fur seal foetuses as well as placentitis in one case (1/11). These reports are in contrast to other pup mortality studies in which only cursory gross necropsies were performed and consequently large proportions (up to 49%) of animals were not able to be designated a cause of death, with a substantial chance of misdiagnosis for those animals with an assigned aetiology (Mattlin, 1978, Reid and Forcada, 2005, McIntosh and Kennedy, 2013). As well as the usefulness of processing post mortem samples for histopathology, this highlights the importance of future NZ sea lion necropsy investigations to include placental

tissue for examination of early neonatal mortality, as well as frozen stillborn tissues for microbiology as this may lead to definitive diagnosis in more cases.

The finding of no gross abnormalities in our small sample of placental tissue is consistent with previous work by Duncan et al. (2012) in northern fur seals but several reports that describe pinniped placental histopathology do not comment on gross findings (Goldstein et al., 2009, Lynch et al., 2011a). Interestingly in the northern fur seals there were no gross signs of placental disease even in those that microscopically were diagnosed with placental infection caused by *Coxiella burnetii*. Due to the focal nature of *C. burnetii* lesions, histopathology alone cannot rule out infection, and further testing with PCR should be undertaken (Kersh et al., 2012). There was no evidence of mineralisation in the NZ sea lion placentas as was seen in 41% of placentas examined from northern fur seals (Duncan et al., 2012). Due to logistical constraints, we were not able to collect placentas from any unhealthy live born, stillborn or aborted pups, which may have increased the chance of finding abnormalities, but this could be improved in the future.

Although alternative to reports in other otariid species, in which disease has been a prominent feature of stillborn pathology, for the initial disease agents of interest it is not surprising that evidence was not found in the NZ sea lion pups examined. Previous work has shown that sub-Antarctic NZ sea lion populations have less than 1% seroprevalence to the agents *Brucella abortus* and *Leptospira interrogans* serovar pomona (Roe et al., 2010) as well as no detectable antibodies to *T. gondii* (S Michael, Chapter Three) indicating that the adult females sampled had not been exposed. Similarly, mainland New Zealand is thought to be one of the few places in the world free of *Coxiella burnetii* (Anonymous, 2010) and this status likely also applies to its outlying sub-Antarctic islands. Nevertheless, this study forms an important baseline for further investigation into late gestational reproductive failure in the NZ sea lion, which should be evaluated as part of assessing the causes of the ongoing

decline of the species. This data can be used to inform future protocols for collection of placental tissue and microbiological samples to improve detection of disease when present and surveillance of emerging infectious agents in the future.

Chapter Three.

Seroprevalence of *Toxoplasma gondii* in mainland and sub-Antarctic New Zealand sea lion populations

3.1. Abstract

Toxoplasma gondii is an emerging risk to marine mammals globally and is known to be present in New Zealand waters. Archived sera of New Zealand sea lions (*Phocarctos hookeri*) from two recolonising mainland populations at the Otago Peninsula and Stewart Island as well as a declining population at Enderby Island in the New Zealand sub-Antarctic were tested for antibodies to *T. gondii*. Sera were screened using commercially available ELISA and latex agglutination tests (LAT) and then confirmed with western blot analysis. Antibodies were found in 13.6% (3/22) of mainland samples but not in sub-Antarctic samples. The positive LAT titres in three adult females were strong (1:2048-4096) and in two of these animals persistent for up to two years, but there was no evidence of associated clinical signs or reproductive failure. Continuing surveillance is pertinent to assess subclinical and clinical impacts of *T. gondii* on these threatened populations.

3.1.1. ***Keywords***

ELISA, latex agglutination test, pinniped, *Toxoplasma gondii*, western blot analysis.

3.2. Introduction

Infectious disease is a threat to pinnipeds and many other wild animal populations that in many cases has not been well investigated. Recently, there has been increased interest in this area, particularly in endangered species including the ecological impacts of disease and their effect on population dynamics. Impacts of disease can range from mass mortality in naïve populations to subclinical effects that suppress population growth and resilience (Woodroffe, 1999). Both ends of this spectrum may be contributing to the ongoing decline of the threatened New Zealand (NZ) sea lion (*Phocarctos hookeri*). Estimated reproductive rates for this species are amongst the lowest for otariids (eared seals; Childerhouse et al., 2010) but male and female fertility, early embryonic development, gestation, parturition and neonatal survival have not been well examined. Infection with the protozoal parasite *Toxoplasma gondii* has been shown to be able to impact all of these developmental stages in mammals and result in decreased reproductive success (Dubey, 2010). Understanding the prevalence of exposure of NZ sea lions to this widespread pathogen may provide an indication of its potential impact on reproduction.

Over 99% of NZ sea lion breeding occurs in the NZ sub-Antarctic between 50-53°S at primary sites in the Auckland Islands and Campbell Island (Figure 3.1; Chilvers et al., 2007b). At the largest colonies of Dundas Island and Sandy Bay, Enderby Island in the Auckland Island group, pup production has been declining consistently. Current estimates report almost half as many pups born annually compared to 1998 when pup production was at an apparent peak (Childerhouse et al., 2014). As a result of the species' restricted range and ongoing decline, it has been classified as 'nationally critical' according to the NZ threat classification system (Baker et al., 2010) and 'vulnerable' by the International Union for the Conservation of Nature (Gales, 2008). Within the last 25 years, there has been gradual recolonisation of mainland NZ including the Otago region (Otago Peninsula and adjacent Catlins coast) as well as southern Stewart Island. The latter now forms the largest breeding area outside of the sub-Antarctic, although still contributing less than 1% of total pup births (B. L.

Chilvers pers. comm). Causes of decline have been postulated to include disease epizootics and interactions with fisheries (Robertson and Chilvers, 2011). Additionally, the species' poor reproductive success may inhibit the capacity for recovery of critical declining populations and the rate of growth of recolonising populations.

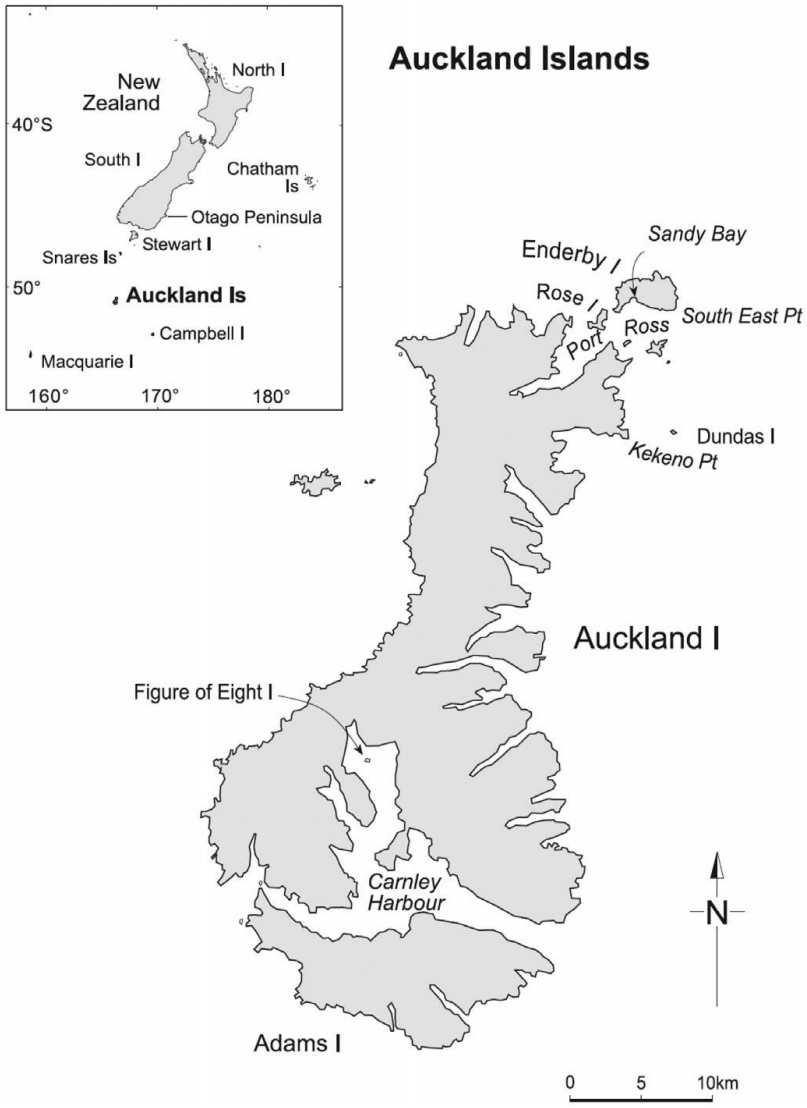

Figure 3.1 Map of New Zealand mainland and sub-Antarctic islands showing New Zealand sea lion (*Phocarctos hookeri*) breeding sites and sampling areas of the Otago Peninsula, Stewart Island and Enderby Island. Image credit: Department of Conservation, New Zealand.

Toxoplasma gondii is emerging as an important pathogen of many species around the world, causing direct mortality as well as indirect effects on reproductive failure (infertility), reproductive loss

(abortion and congenital foetal defects) and behavioural changes which can increase predation risk (Afonso et al., 2012, Stahl et al., 1994, Elmore et al., 2010). *T. gondii* is a cosmopolitan apicomplexan parasite that is widely prevalent and able to infect any warm-blooded host including humans via felid definitive hosts (Tenter et al., 2000). Although only felids are capable of shedding *T. gondii* oocysts into the environment (Elmore et al., 2010), it is increasingly evident that exposure to the parasite, clinical disease and death due to toxoplasmosis is also prevalent in marine mammals globally (Jardine and Dubey, 2002, Bossart et al., 2012, Donahoe et al., 2014). Particularly, this has been intensively studied in southern sea otters (*Enhydra lutris nereis*) in California, USA where encephalitis due to *T. gondii* infection has been declared the primary cause of death in 16.2% (17/105) of otters and a contributing factor in another 11.4% (12/105) of deaths over a three year period in a declining population (Kreuder et al., 2003). Additionally, recent work on Hector's dolphins (*Cephalorhynchus hectori*) in NZ waters has shown that disseminated toxoplasmosis accounted for 25% (7/28) of deaths investigated over a four year period (Roe et al., 2013).

While the aforementioned marine host species seem particularly susceptible to clinical disease and death following *T. gondii* infection, antibody response indicating exposure to *T. gondii* in apparently healthy hosts has been described in a wide range of marine mammal species including cetaceans, pinnipeds and sirenians in most regions of the world, including Antarctica (eg. Dubey et al., 2003, Rengifo-Herrera et al., 2012, Sulzner et al., 2012). Amongst otariids, seroprevalence has been reported in California sea lions (*Zalophus californianus*; 30-61%; Dubey et al., 2003), Steller sea lions (*Eumetopias jubatus*; 0-83%; Burek et al., 2003), Antarctic fur seals (*Arctocephalus gazella*; 0-2.4%; Rengifo-Herrera et al., 2012, Tryland et al., 2012) and Australian fur seals (*Arctocephalus pusillus doriferus*; 0%; Lynch et al., 2011b). Several techniques for measuring antibody titres have been reported including the direct agglutination test (DAT), modified agglutination test (MAT), indirect latex agglutination test (LAT), indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) (Opel et al., 1991, Dubey et al., 2005). Each test has advantages and disadvantages however comparisons in seroprevalence levels between populations is problematic,

with several studies using different threshold values for the same test and species assessed (Jensen et al., 2012, Rengifo-Herrera et al., 2012).

Within New Zealand, serological investigations in marine mammals are lacking, however infection with *T. gondii* has long been recognised as a common cause of abortion in small ruminants (Opel et al., 1991, West, 2002). Clinical or necropsy evidence of toxoplasmosis has not been reported in NZ sea lions, however histopathology and molecular techniques that are required for post mortem diagnosis are often not undertaken. Seroprevalence indicating exposure to *T. gondii* has not been investigated to date. The aim of this study was to provide baseline information by retrospectively assessing seroprevalence of mainland and sub-Antarctic NZ sea lions to *T. gondii* using two commercially available antibody tests, validated with western blot analysis.

3.3. Materials and methods

Total NZ sea lion population size was estimated at 144 for Otago Peninsula (Anonymous, 2014, McConkey et al., 2002a), 150 for Stewart Island (B. L. Chilvers pers. comm.) and 1080 for Enderby Island (B. L. Chilvers pers. comm; Childerhouse et al., 2014). The sample size (n) necessary to detect *T. gondii* in respective NZ sea lion populations was calculated using the EpiTools sample size for freedom testing calculator (Sergeant, 2014c). Accordingly, minimum numbers of serum samples from Otago Peninsula (n=10), Stewart Island (n=10) and Enderby Island (n=11) required analysis in order to detect the presence of *Toxoplasma gondii* at an expected minimum prevalence of 29.67% (mean prevalence detected in otariids; Dubey et al., 2003, Burek et al., 2003, Tryland et al., 2012, Lynch et al., 2011b) at a 95% confidence level. Archived sera stored at -80°C were available from previous live captures of NZ sea lions at the Otago Peninsula between 2008-2010 (46°00'S, 170°40'E; n=15, from 10 animals), Stewart Island between 2012-2013 (47°00'S, 168°00'E; n=12, from 12 animals) and Enderby Island between 2009-2012 (50°30'S, 166°17'E; n=28, from 28 animals). From the Otago Peninsula, five individuals had dual samples collected one to two years apart. Samples were from adult females, except for one adult male and six juveniles (1-3 years).

All sera (n=55) were tested according to manufacturer's instructions using a *T. gondii* enzyme-linked immunosorbent assay (ELISA) developed for small ruminants (Chekit™ Toxotest, Idexx Laboratories, Liebefeld-Bern, Switzerland) and an indirect latex agglutination test (LAT) (Toxoreagent, Eiken Chemical Company, Tokyo, Japan). No official interpretation guidelines exist for these tests for use with marine mammal sera. Manufacturer's interpretation for small ruminants using the ELISA was 'suspect' if S/P ratio values were between 20-30% and 'positive' if greater than 30%. For the LAT, titres greater than 1:32 were considered 'positive' and this threshold was used for NZ sea lion sera. All suspect, positive and disparate results between these two tests as well as several negative results were confirmed with western blot as described below.

3.3.1. *Preparation of water-soluble* **T. gondii** *antigen for western blot*

Live tachyzoites of an attenuated strain (S48) of *T. gondii* from a vaccine (Toxovax, MSD Animal Health, Upper Hutt, New Zealand) were used to extract antigen. Thirty millilitres of vaccine suspension was centrifuged at 1500 x g for 10 minutes to pellet tachyzoites. The pelleted tachyzoites were re-suspended in 2ml PBS (pH 7.4) and disrupted by 3 cycles of freezing and thawing. This was followed by 7 cycles of sonication on ice (Sonics Vibracell, Sonics & Materials Inc., Newtown, CT, USA). The sonicated tachyzoites were centrifuged at 12,000xG for 30 minutes at 4°C to remove debris and the supernatant containing the water soluble proteins was collected. Protein concentration of the antigen supernatant was measured with spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE, USA). Successful protein preparation was confirmed using standard SDS-PAGE protocols (Appendix 5).

3.3.2. *Western blot*

Antigen (0.75 µg/µL) was mixed with an equal volume of 2x Laemmli buffer (Bio-Rad, Hercules, CA, USA) and denatured by heating at 100°C for 5 minutes. The denatured protein (48µg total antigen/gel) and a protein marker with a molecular weight range of 10-250kDa (Precision Plus Protein Kaleidoscope Standards, Bio-Rad) was run through a 12.5% Tris-HCl gel (Criterion Prep+2, Bio-Rad) for 90 minutes at 120V in a Tris-glycine SDS buffer containing 25mM Tris, 192mM glycine and 0.1% (w/v) SDS (pH 8.3) (Bio-Rad). After separation, the proteins were electrophoretically transferred onto a PVDF membrane (Immun-blot, Bio-Rad) for 60 minutes at 60V in a Tris-glycine buffer containing 25mM Tris, 192mM glycine and 20% w/v methanol (pH 8.3) (Bio-Rad). After confirmation of protein transfer through Ponceau S staining (Sigma, St Louis, MO, USA), the membrane was blocked overnight at 4°C using a 5% blotting solution consisting of 5% skim milk powder in phosphate buffered saline with 0.05% Tween-20 (Bio-Rad). Blocked membrane was cut into strips and incubated with test sera as well as positive and negative control sera, diluted 1:100 in

blocking buffer at room temperature for 120 minutes. After primary incubation, strips were washed three times for 10 minutes using PBS-0.05% Tween-20 solution. Secondary generic antibody (purified recomb protein A/G peroxidase conjugated, Thermo Scientific) diluted 1:5000 in 5% blotting solution with 0.05% Tween-20 was added to each strip and incubated for 60 minutes at room temperature. Strips were washed again three times as described above, incubated for five minutes in chemiluminescent solution (Clarity Western ECL substrate, Bio-Rad) and arranged on a transparent plastic film. The film was exposed for 30 seconds in a luminescent image analyser (LAS-1000 plus, Fujifilm, Tokyo, Japan) and the bands were measured against standard markers. Sera that recognised two or more immunodominant antigens of *T. gondii* with molecular weight between 13-48kDa were considered positive.

3.3.3. ***Statistics***

For individuals with dual samples, only their most recent result was included in statistical analyses. The probability of *T. gondii* being present in NZ sea lion populations at each site, according to the results of the LAT, was calculated based on a hypergeometric exact probability formula by Cameron and Baldock (1998) using the FreeCalc V.2 software (Sergeant, 2014b). The sensitivity and specificity used for the Eiken LAT were averaged from several reports in human subjects at 95% and 89.8% respectively (Johnson et al., 1989, Woldemichael et al., 1998, Evans and Ho-Yen, 2000). The Epitools epidemiological calculator was used to estimate the true prevalence and its 95% confidence intervals using Blaker's exact confidence limits for the Eiken latex agglutination diagnostic test (Reiczigel et al., 2010, Sergeant, 2014a). A P value <0.05 was considered significant.

3.4. Results

3.4.1. *LAT, ELISA and western blot analysis*

There was an aligned pattern between the results of LAT and ELISA methods. Based on the small sample size analysed here, three discrete groups were evident amongst the NZ sea lion sera tested with the LAT: those with very strong reactions (n=5; LAT ≥1:2048), weak reactions (n=12; LAT 1:16-32,) and no reaction (n=38; LAT =0) (Table 3.1, Appendix 6). ELISA results were well associated in the strong reaction and no reaction groups, but results in the weak reaction group were more poorly defined with ELISA. Western blot was used to evaluate the antibody reactivity of a subset of 14 samples. Five sera in the strong reaction group were confirmed to be positive (n=5) and four sera in the no reaction group were confirmed to be negative (n=4). However, the five samples in the weak reaction group produced a banding pattern not consistent with *T. gondii* and it is likely that these were mild cross-reactions (Figure 3.2).

Table 3.1 Comparison of LAT and ELISA result ranges for confirmed positive, cross reaction and negative New Zealand sea lion (*Phocarctos hookeri*) serum samples on *T. gondii* western blot analysis.

Western blot result	Sample size[a]	LAT Titre Range	ELISA S/P Value Range (%)
Positive	5	1:2048-1:4096	19.1-27.7
Cross reaction	5	0-1:32	0.3-13.6
Negative	4	0-1:16	-2.2-0

[a]Number of serum samples (includes samples collected from an individual in different years).

Western blot results indicate that the manufacturer's threshold ranges for the small ruminant developed ELISA are not relevant for NZ sea lion serum. At an S/P ratio threshold of >30% for positive, all western blot positive sera tested would have been interpreted as ELISA negative or suspect (S/P ratios of 19.1%, 20.1%, 20.5%, 24.9% and 27.7%). This suggests that a proposed lower threshold of approximately 16% is recommended when considering sera reactivity with NZ sea lion samples and further refinement with testing of additional samples is required. On the other hand, the threshold values developed for the Eiken LAT appear to be accurate for use with the sample of NZ sea lion sera tested in this study. However, since no moderate reactions (LAT 1:64-1024) were

identified and only a small sample was available for analysis, these thresholds as well as the test sensitivity and specificity cannot be further defined without additional samples.

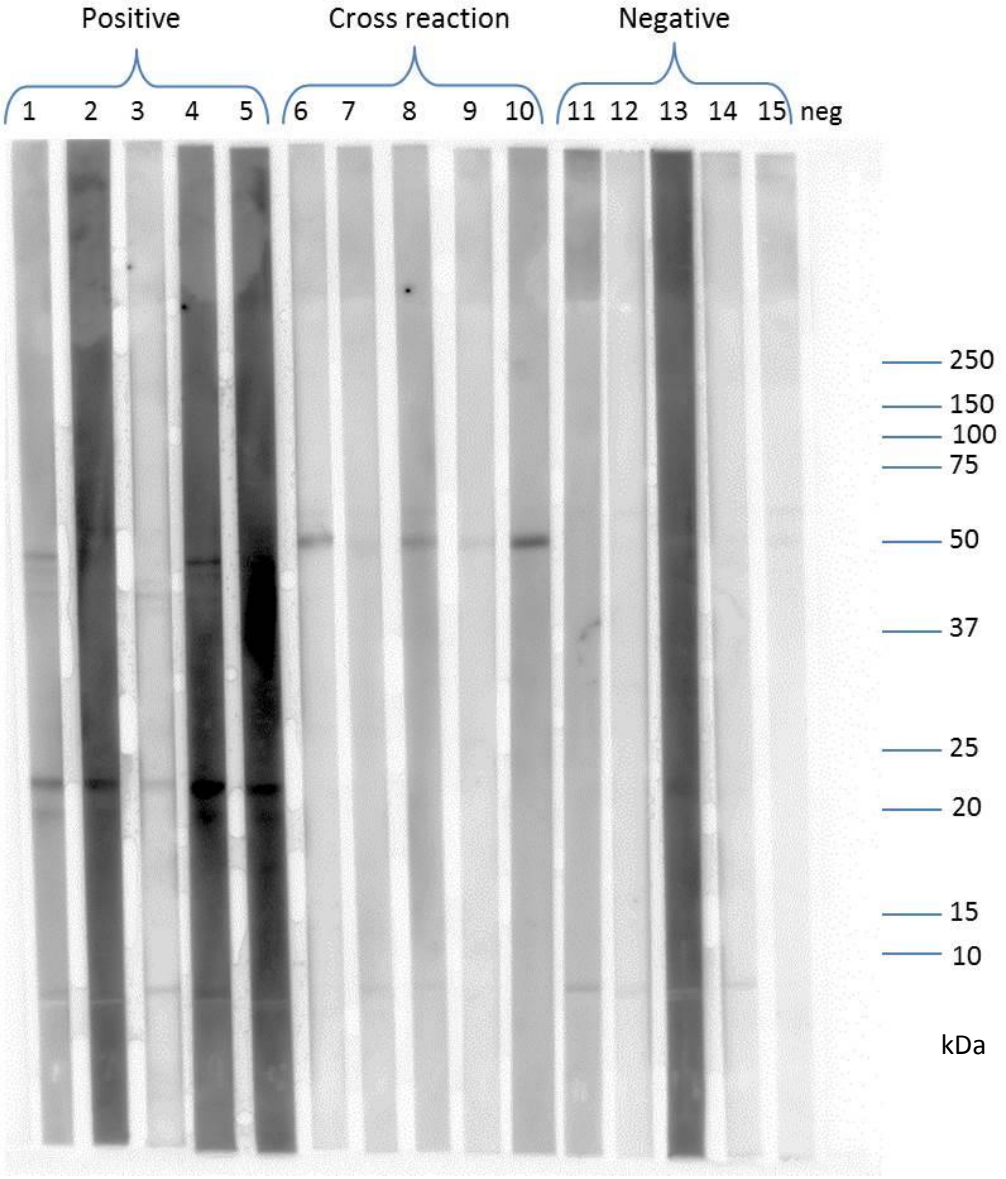

Figure 3.2 Western blot analysis for *Toxoplasma gondii* antibodies in New Zealand sea lion (*Phocarctos hookeri*) serum with protein standard on right, showing positive, suspected cross reaction and negative samples. neg, negative control

The five confirmed positive samples were from three adult females from mainland locations. Two of these animals from the Otago Peninsula exhibited high LAT antibody titres (1:4096, 1:2048) in both occasions of being sampled one and two years apart respectively. The other animal was from Stewart Island and was only sampled once with a LAT titre of 1:4096. No animals from Enderby

Island were found to be positive. All identified cross reactive samples on western blot were from Enderby Island animals.

3.4.2. ***Statistics***

Using the hypergeometric exact probability formula and LAT test results, *T. gondii* was found statistically to be present in the Otago Peninsula population of NZ sea lions (P=0.24), but absent from the Enderby Island (P<0.0001) and Stewart Island (P=0.03) populations at the minimum expected prevalence of 29.67%. This is reflected in estimates of true prevalence (Table 3.2), showing that *T. gondii* exists at low levels in mainland NZ sea lion populations but the sub-Antarctic populations tested are free of the pathogen.

Table 3.2 Apparent and true prevalence of *Toxoplasma gondii* in New Zealand sea lion (*Phocarctos hookeri*) populations in mainland and sub-Antarctic locations determined using the Eiken latex agglutination test.

Site	Positive/tested	Apparent prevalence (%)	95% confidence interval[a]	Estimated true prevalence (%)	95% confidence interval[a]
Otago Peninsula	2/10	20	5.7-51	11.6	0-53.5
Stewart Island	1/12	8.3	1.5-35.4	0	0-31.1
Enderby Island	0/28	0	0-12.1	0	0-1.4
All sites	3/50	6	2.1-16.2	0	0-7.3

[a]Confidence intervals for true prevalence determined using Blakers analysis. Sensitivity: 95%; specificity: 89.8%.

3.5. Discussion

This is the first report of *Toxoplasma gondii* seroprevalence in NZ sea lions and suggests exposure of mainland but not sub-Antarctic sample to the parasite. Further, the ELISA and LAT tests were shown to produce accurate results with NZ sea lion serum and with further refinement, either could be an inexpensive rapid test to monitor prevalence in populations sampled opportunistically. As Otago Peninsula and Stewart Island populations continue to gradually recolonise in the presence of large feral cat populations (Moller and Alterio, 1999, Harper, 2005) and sub-Antarctic populations remain on a downward trajectory, it is important to monitor the subclinical and clinical impacts of the parasite on NZ sea lion health and reproduction.

Apparent seroprevalence to *T. gondii* was within the lower end of the ranges reported for other otariids. Both commercial tests were well correlated, however the threshold for the ELISA was not accurate for the sea lion samples tested with western blot. This is likely because the ELISA was developed for small ruminants and there could be poor reactivity with NZ sea lion serum and weaker results. On the other hand, the LAT is a non-species specific test and accordingly, positive and negative samples tested with western blot related well to LAT results. A proposed lower threshold of 16% for the ELISA would provide an improved fit for validated test results and with further samples tested would become more reliable. The cause of the cross reactions in a subset of samples from current testing was not clear. Protozoa that are closely related to *T. gondii* including *Neospora caninum* and *Sarcocystis* spp. are potentially capable of causing this result. *Sarcocystis* spp. has been observed in skeletal muscle sections on histopathology in NZ sea lions previously (B. Lenting and W. D. Roe pers. comm.) so it is reasonable to assume some of the animals in this study may have been exposed. *N. caninum* has not been reported in any NZ wildlife species, but is known to be harboured in NZ dogs, commonly causing epidemic abortions in cattle (Reichel, 2000).

The clinical relevance of persistently high antibody titres seen in the two Otago Peninsula females is unclear and difficult to assess due to the large interval between samples (1-2 years). Similarly, it is

difficult to draw conclusions from a single positive result as in the case of the Stewart Island female, except that the animal had been exposed at some point in time. Confirmation of an active infection in humans and domestic species generally requires serial samples 3-4 weeks apart and demonstration of at least a four-fold rising titre (Montoya, 2002). Unfortunately these samples are not available for NZ sea lions. The LAT detects all anti-*Toxoplasma* antibody, while the ELISA in ruminants at least, is specific to IgG. In humans, diagnostics are complicated by often prolonged IgM and IgG responses, which in immunocompetent individuals can persist for over a year and up to ten years respectively following infection with *T. gondii* as an adult (Remington, 1974, Montoya, 2002). Similarly very high antibody titres 14 months apart were reported from an urban common brushtail possum (*Trichosurus vulpecula*) without any obvious clinical signs, even though toxoplasmosis has been shown to cause fatal disseminated disease in this species (Canfield et al., 1990, Eymann et al., 2006). Additional testing for IgM titres and IgG avidity may be useful in determining whether these animals are experiencing multiple acute or chronic infections, however further species validation would be required. With the present stage of validation of the LAT and ELISA, testing of NZ sea lions can be efficient and inexpensive, allowing for more current and opportunistic seroprevalence levels especially in at-risk mainland locations.

T. gondii is well known to cause reproductive loss when infection occurs during pregnancy, however this did not appear to be the case at least in the two persistent strongly seropositive Otago Peninsula females as both were observed to deliver normal pups in the seasons of and following serum collection (Anonymous, 2014). This is consistent with reports in female western grey kangaroos (*Macropus fuliginosus ocydromus*) where seropositivity to *T. gondii* was not related to impaired fertility or reproductive performance (Mayberry et al., 2014). The gestation period of NZ sea lions is not known but thought to be approximately 9 months following a presumed embryonic diapause of 3 months (Boyd, 1991), so infection at almost any time of the year could impact on the developing foetus. Reproductive failure is rarely reported in marine mammals, generally only in stranded pregnant females or neonates and diagnosed at post mortem (eg. Resendes et al., 2002, Miller et al.,

2008). The true incidence is unknown as abortions may occur at sea or neonates with encephalitis or congenital defects could be easily predated before being identified. In addition to vertical foetal transmission and infection, *T. gondii* tissue cysts in uterine tissue causing metritis have been identified in a Hector's dolphin and such pathology may create a hostile environment for growth and maintenance of a foetus (Roe et al., 2013). Due to the female-biased selection of samples available for testing, most emphasis has been placed on the female effects of *T. gondii* infection, however experimental infection in rodents has shown that the parasite is able to induce infertility in both males and females (Stahl et al., 1994, Terpsidis et al., 2009). Although individual male fertility is likely to be of low importance in the overall fecundity of pinnipeds with a polygynous breeding strategy, the wide geographic dispersal of males within and between breeding seasons could increase their exposure to oocysts in the environment and this cohort may serve as a more potent indicator of exposure and disease, albeit more practically difficult to sample (Robertson et al., 2006).

Feral cats are currently present throughout most of the terrestrial range of the NZ sea lion including all those sampled in this study. Domestic cats were introduced to New Zealand by early explorers and settlers in the 1770s, eventually dispersing and establishing feral populations throughout the mainland as well as on offshore islands (King, 1984). As there are no native felids present in NZ, all environmental contamination with *T. gondii* oocysts is derived from roaming domestic and feral house cats. On the mainland there are large populations throughout the Otago Peninsula (Moller and Alterio, 1999) and Stewart Island (Harper, 2005). In the sub-Antarctic, there are feral cats present on the main Auckland Island (less than 10km away from Enderby and Dundas Islands), becoming established about 1820, however felids were never introduced to any other islands in the archipelago (Harper, 2010). Scant numbers present on Campbell Island were eradicated in 2001 (Convey and Lebouvier, 2009). The prevalence of *T. gondii* infection in these Auckland Island cat populations is unknown but at least in mainland cats is likely to be high (Thompson, 1993), which may be reflected in the spatial distribution of the positive serum samples analysed here.

Reduction of feral cat populations is underway in some localities of the mainland but contamination of the environment with sporulated oocysts can be prolonged as they can persist in soil for at least 18 months (Frenkel et al., 1975). In addition, oocyst-contaminated freshwater runoff has been strongly implicated in the delivery of the parasite to the marine environment, where sporulated oocysts have been shown to remain infectious after two years in sea water at 4°C (Miller et al., 2002, Lindsay and Dubey, 2009). Once in the ocean, oocysts may become bound to aggregates that preferentially adhere to vegetation or sediment at the benthos (Shapiro et al., 2012). This presumed distribution of oocysts is important as benthic invertebrates may consume the oocysts, hence acting as vectors to deliver infectious material to higher trophic levels of the food chain (Shapiro et al., 2012). Accumulation of infective oocysts by bivalve molluscs and filter-feeding fish has been demonstrated (Arkush et al., 2003, Lindsay et al., 2004). While NZ sea lions are opportunistic generalist feeders, both the mainland and sub-Antarctic location diets are not known to consist of significant proportions of invertebrates or filter-feeding fish (Meynier et al., 2009, Augé et al., 2012b). However, it is not known if ingestion of these prey items by larger fish that are part of the NZ sea lion diet would result in infection with *T. gondii* or seroconversion. Furthermore, Otago NZ sea lions and a subset of the Auckland Islands population forage in a benthic pattern and may incidentally ingest sediment containing oocysts (Chilvers and Wilkinson, 2009, Augé et al., 2011).

All the serum samples used for this study were collected as part of a foraging study employing satellite and time-depth recorders to determine foraging type and areas utilised (Chilvers and Wilkinson, 2009, Augé, 2010). Since no samples from Enderby Island animals were positive, we are unable to compare this effect amongst benthic (bottom diving) and mesopelagic (diving throughout the water column) foraging specialities. The two seropositive females from the Otago Peninsula were identified in this previous work by Augé (2010) as having either spent significantly more time ashore than any other females or the smallest (exclusively coastal) foraging range. Both alternatives could theoretically increase the risk of land-based or coastal runoff exposure to oocysts, but further work is required to assess the level of environmental contamination in critical NZ sea lion habitats.

In conclusion, this study provides the first reported evidence for exposure of mainland NZ sea lions to *Toxoplasma gondii*, although more research is required to evaluate the clinical relevance of this finding and further enhance testing. Whilst only a small sample size was available, this study shows that it is unlikely that sub-Antarctic populations have been exposed to the parasite. Testing of non-breeding adult females and males may help unravel the complex interactions of the parasite and its potential impacts on reproduction. Continuing surveillance of breeding females should become an important monitoring tool to assess subclinical and clinical impacts on these threatened populations.

Chapter Four.

Long term survival and reproductive success of New Zealand sea lions treated with ivermectin as pups

4.1. Abstract

Hookworms (*Uncinaria* spp.) are a common parasite of neonatal fur seals and sea lions around the world and may contribute to decreased growth and survival. Removal of these parasitic burdens by administration of the anthelmintic ivermectin has been trialled in New Zealand (NZ) sea lion (*Phocarctos hookeri*) pups at Sandy Bay, Enderby Island with initial benefits reported in growth and survival, however long term effects on survival and reproduction have not been previously examined. Resighting data was assessed with the Cox proportional hazards analysis to evaluate survival to maturity and fecundity of treated and control pups from the 2002-2004 study. Sample size was a limiting factor as juvenile survival was very low but a trend of improved survival was observed in the ivermectin-treated group over the 8-9 year period assessed. The year of birth of the pups had a significant effect due to a bacterial epizootic in the first year of the trial. Reproductive rate was not significantly different between groups. Further research is warranted to investigate drivers of the high first year mortality evident in the study and mitigation techniques such as anthelmintic treatment for improving survival and recruitment in declining populations.

4.1.1. ***Keywords***

Hookworm, ivermectin, management, pinniped, survival, survival analysis, *Uncinaria.*

4.2. Introduction

Hookworm (*Uncinaria* spp.) infestation is a commonly reported cause of morbidity and mortality in neonatal pinnipeds, particularly otariids (fur seals and sea lions) around the world (Lyons et al., 2011b). In severe cases, pups can suffer from enteric mucosal haemorrhage and consequent anaemia, weight loss, weakness and death. Elimination of these worm burdens in affected wild pinniped populations by the administration of parenteral anthelmintics has been advocated as a potential management tool to improve pup growth and survival, especially when survival may also be under influence of other factors such as bacterial infections (Chilvers et al., 2009). The effect of removal of worm burdens on improving weight gain and reproductive performance in domestic animals has been established (Forbes et al., 2002, Loyacano et al., 2002) and the optimisation of neonatal nutrition and growth in wild animal populations has been shown to be linked to subsequent improved adult fitness and fecundity (Lindström, 1999, Metcalfe and Monaghan, 2001). Despite the potential benefits of anti-parasitic treatment to positively influence early development, with apparent downstream advantages for future survival and breeding success, little is known about such outcomes in wild pinniped populations.

The New Zealand (NZ) sea lion (*Phocarctos hookeri*) is a rare and highly localised otariid, with a distribution predominantly encompassing the NZ sub-Antarctic islands between 50-53°S. Around 73% of the pup production for the entire species occurs on two islands (Dundas and Enderby Islands) less than 10km apart, within the Auckland Island archipelago (Figure 4.1; Maloney et al., 2012). The species' pup production is in decline, now at approximately 50% of previous estimates reported when consistent study commenced in 1998 (Childerhouse et al., 2014), leading to threat classifications of 'threatened' by the International Union for Conservation of Nature (Gales, 2008) and 'nationally critical' in New Zealand (Baker et al., 2010). The cause of the population decline is likely to be multifactorial with disease, incidental death, bycatch and competition with fisheries being potential contributors (Robertson and Chilvers, 2011). Recent modelling research has

suggested that low pup and yearling survival is perpetuating the ongoing decline (Roberts et al., 2013), which may be in part driven by clinical disease in the form of periodic bacterial epizootics as well as subclinical disease such as hookworm burdens with subsequent low grade anaemia. The second largest and most intensively studied breeding colony at Sandy Bay, Enderby Island has been subject to several mass pup mortality events caused by *Klebsiella pneumoniae* which have resulted in up to 33% pup mortality in the most severely affected season (Wilkinson et al., 2004). Previous studies have also shown that up to 100% of pups at Sandy Bay are infested with *Uncinaria* spp. by the end of January (pups on average 1 month old; Castinel et al., 2007a) and hookworm haemorrhagic enteritis was a significant finding on post mortem for approximately 13% of dead pups between 1998 and 2005 (Castinel et al., 2007b).

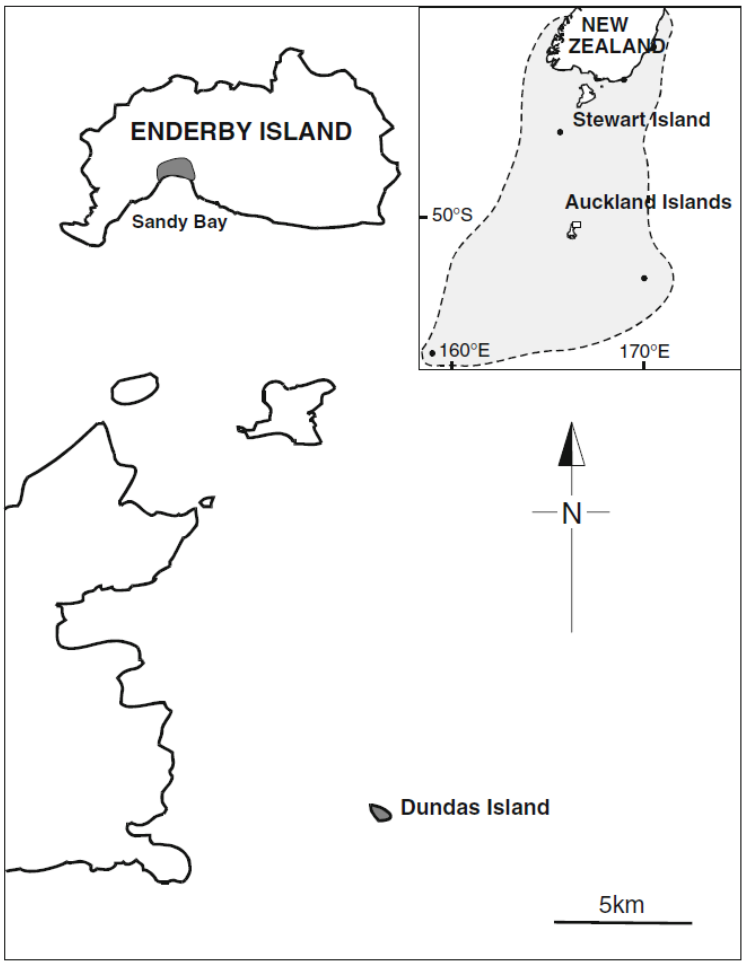

Figure 4.1 Map of northern Auckland Islands showing Sandy Bay and Dundas Island breeding colonies. Inset shows current known distribution of New Zealand sea lions (*Phocarctos hookeri*; shaded). Image credit: Department of Conservation, New Zealand.

Extensive research has been undertaken into the biology of hookworms primarily in northern fur seals (*Callorhinus ursinus*; Lyons et al., 2011b), and it is likely that a similar situation applies for NZ sea lions (Castinel et al., 2007a). Pups, the definitive hosts for *Uncinaria* sp. acquire stage 3 larvae (L3) from their mothers as newborns through ingestion of infected colostrum (Olsen and Lyons, 1965). The larvae mature in the small intestine of pups with a prepatent period of 2-4 weeks (Castinel et al., 2007a). After this time, eggs are passed in faeces and parasites mature to free-living L3 stage, particularly on sandy substrates. These L3 are acquired by adult sea lions either percutaneously or orally but do not mature further, rather migrating to the ventral abdominal blubber and becoming dormant. These have been shown to be viable for up to six years in northern fur seals (Lyons et al., 2011b). Adult male sea lions are a dead end hosts and show no demonstrable clinical effects from the subcutaneous larvae, however in pregnant females, the larvae are reactivated around the time of parturition to migrate to mammary tissue and infect the next generation of pups (Lyons et al., 2011b). Pups are only thought to be infected for the first half of the period spent on land after birth, the most advantageous time for parasite transmission- around 3 months in northern fur seals or 6-8 months in California sea lions (*Zalophus californianus*; Lyons et al., 2000). Infection in pups appears to be self-limiting as infective larvae are only passed for a short time in milk and pups do not establish a patent infection by other routes.

Although anthelmintics are used routinely in captive pinnipeds (Stoskopf et al., 2001), little research has been reported on the effects and practicalities of dosing wild populations. A study in northern fur seals (DeLong et al., 2009) showed apparent increase in growth rate and early survival in pups treated with ivermectin (a broad spectrum avermectin anthelmintic). A similar study in NZ sea lion pups undertaken by Chilvers and colleagues (2009) reported increased growth rate in treated pups but not significantly improved survival except during a season concurrently affected by a bacterial epizootic event. Although these studies address the immediate effects of clearing a worm burden, the longer term implications on survival to adulthood and reproduction have not been reported. Further, the environmental fate of avermectins in the terrestrial and marine ecosystems occupied by

medicated animals requires careful consideration as these compounds have been demonstrated to have unintended negative effects beyond the host, particularly on invertebrates (Wall and Strong, 1987, Sanderson et al., 2007, Liebig et al., 2010). Continuing use of ivermectin to assist in NZ sea lion conservation necessitates clear demonstration that the benefits to this threatened species outweigh the environmental costs of its use. The aim of this research was to retrospectively assess if there was any long term improvement in survival and fecundity in the sample of NZ sea lion pups dosed with ivermectin and their controls 8-9 years after treatment.

4.3. Materials and methods

Selection and treatment of pups was carried out as described in Chilvers et al. (2009) in the 2002/03 and 2003/04 austral summer breeding seasons at Sandy Bay, Enderby Island (50°30'S, 166°17'E). Briefly, 40 pups (20 treated and 20 untreated) in 2002/03 and 44 pups (22 treated and 22 untreated) in 2003/04 were selected at birth and matched within groups for the parameters of gender, birth date and birth mass. Treated pups were administered 0.2mg/kg ivermectin (Ivomec® Injection for Cattle, Sheep and Pigs, Merial Ancare, Manukau City, New Zealand) subcutaneously in the interscapular region at 3, 7 and 30 days of age. Control pups were not sham injected with saline. All pups were initially identified by marker caps glued to the head, then later tagged with coffin shaped plastic tags (Jumbotags, Dalton ID Systems Ltd, Oxon, England) medially on the trailing edge of both pectoral flippers. All pups were weighed and measured regularly throughout each study period of 93 days and pups that died during the time when researchers were present underwent necropsy to determine cause of death.

Resighting of flipper tags was conducted at Sandy Bay daily and opportunistically at other locations between approximately 1st December and 20th February each season between 1998 and 2012. All field resighting data was entered into a database and several criteria were applied to reduce bias from tag misreading: 1) animals were marked as resighted for a particular year if they were positively identified (by tag) at least three times within that year, 2) animals identified less than three times within a year were counted as not resighted, 3) females were marked as having pupped if resighted at least three times in the presence of or nursing a particular pup and 4) females seen less than three times with a particular pup were counted as not having pupped. All animals were then assigned a status of either 'confirmed dead' if the animal had been resighted dead, 'assumed dead' if the animal had not been resighted for the preceding five years or 'assumed alive' if the animal had been positively identified in the preceding five years.

Based on these resighting records, a Cox proportional hazards model (Cox, 1972) was run using SPSS version 21 (SPSS IBM, New York, USA) with the covariates of sex, year of birth and ivermectin treatment group. For each animal in the sample total 'days at risk' was calculated based on exact date of birth and either confirmed or assumed date that the animal died or was last seen. The latter was designated to animals in the 'assumed dead' and 'assumed alive' groups as an arbitrary date of 30th June in the year following the last season resighted. Those animals that had been resighted in the most recent season 2011/12 were assigned 30th November 2012 as immediately prior to the next expected resighting period. Due to the high level of uncertainty with this data, best and worst case scenarios were modelled with the idea that the true situation would be somewhere between. Best case scenarios refer to data with the 'assumed dead' category assigned as assumed alive, while in worst case scenarios the 'assumed dead' category is assigned as 'confirmed dead'.

A multivariate logistic regression model was used to identify variables influencing survival outcome (i.e. animals that survived to the end of this study; SPSS IBM). Variables assessed included sex, year of birth and ivermectin treatment group.

Individual female reproductive rate was calculated based on resighting data of number of confirmed pups divided by the number of years in which the individual was four years or older (the earliest expected pupping age except in rare cases (Childerhouse et al., 2010)). These variables were compared using the student's T test with two tails and a P value <0.05 was considered significant.

4.4. Results

Of the 84 pups recruited to the study, eight were recorded as confirmed dead within the monitored period to three months of age, of which six were in the control group and two were in the ivermectin treatment group. Following the initial seasons of birth, there were no confirmed deaths amongst this sample, however 52 of the 76 animals thought to still be alive (68.4%) were never resighted in any of the following years of the study (Figure 4.2). Hence, this large proportion of animals with no confirmed outcome in the assumed dead category may confound the statistical significance of further tests and becomes a prominent feature of worst case survival analysis graphical output.

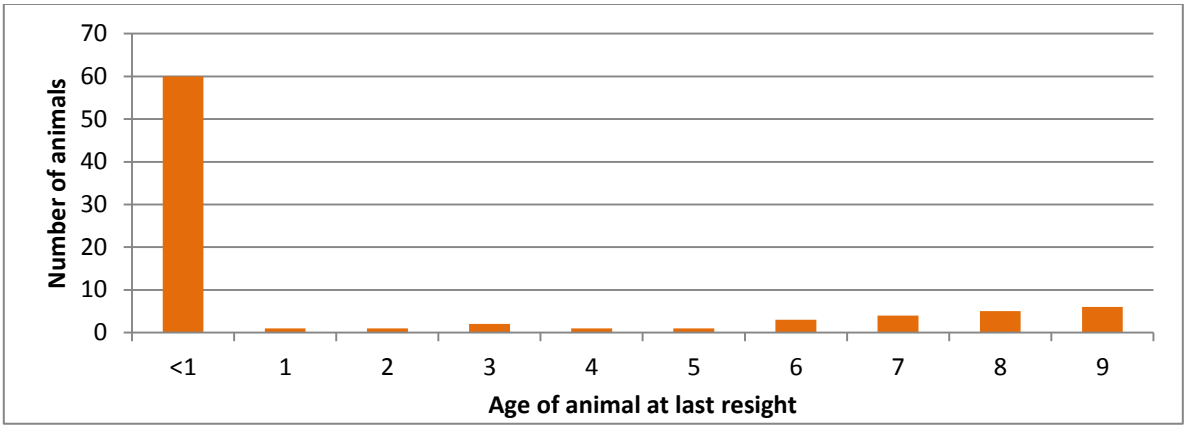

Figure 4.2. Age at last resight for New Zealand sea lion (*Phocarctos hookeri*) pups in ivermectin trials. Treatment and control groups, and 2002/03 and 2003/04 birth year cohorts are combined.

In the best case models the significance of early survival is amplified as no confirmed deaths were identified in older animals. In the worst case models, the data is similarly skewed towards early survival due to the very high proportion of animals not resighted again since the season in which they were born.

4.4.1. *Best case model*

There was a significant year difference in early survival amongst the sample, with pups born in 2003/04 more likely to survive past three months of age than those born in the previous season

(P=0.041; Figure 4.3). There was also an advantage for pups treated with ivermectin and female pups, however the difference was not significant (P>0.05).

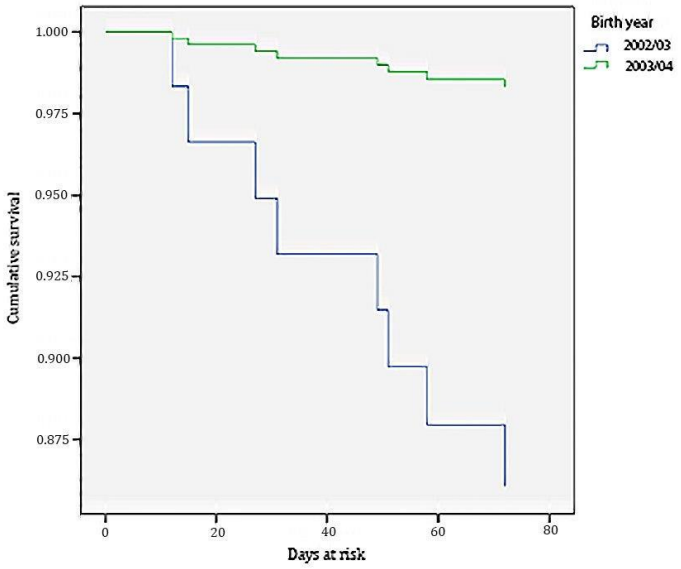

Figure 4.3 Best case survival analysis for New Zealand sea lion (*Phocarctos hookeri*) pups with birth year covariates using a Cox proportional hazards model

An analysis of 2002/03 hazard ratio data generated from the survival analysis showed a corresponding protective effect of female sex and ivermectin treatment, however these were not significant (sex, P=0.081; treatment group, P=0.274; Figure 4.4; Table 4.1). Data from 2003/04 was not analysed due to only one pup from the sample dying during the first three months.

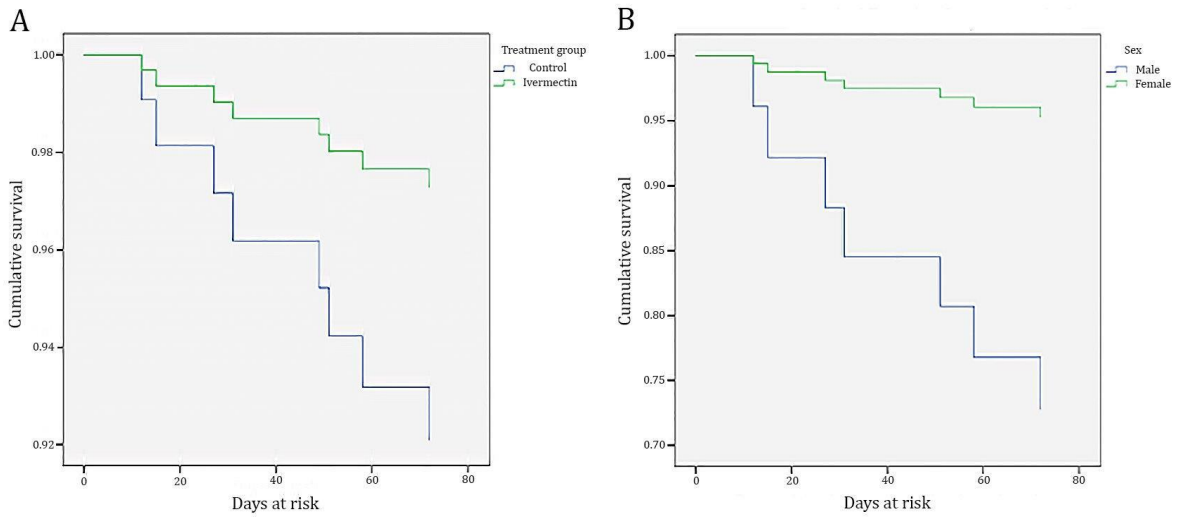

Figure 4.4. Best case survival analysis using a Cox proportional hazards model for New Zealand sea lion (*Phocarctos hookeri*) pups born in the 2002/03 season with covariates of A. ivermectin treatment group and B. sex.

Table 4.1. Comparison of hazard ratio (HR) with 95% confidence intervals (CI) using a Cox proportional hazards model for covariates of ivermectin treatment group and sex in New Zealand sea lion (*Phocarctos hookeri*) pups born in the 2002/03 season.

	Covariate	P value	Hazard ratio	95% CI for HR
2002/03	Ivermectin	0.274	2.497	0.484-12.880
	Sex	0.081	6.571	0.790-54.633

Logistic regression of survival outcome echoed the above results with the odds of survival being 10.8 times higher in 2003/04 compared to the previous year (95% CI 1.201-96.924; P=0.034). Pups treated with ivermectin and female pups were 3.8 and 3.7 times more likely to survive respectively, however this was not statistically significant (treatment group, P=0.134; sex, P=0.138).

4.4.2. ***Worst case model***

Although early survival was significantly worse in 2002/03, survival past one year old tended to be higher than 2003/04 (P=0.287; Figure 4.5a). There were no significant differences between sex or treatment group, however more pups treated with ivermectin survived past their first year (Figure 4.5b). Pups born in 2002/03 survived on average 1092 days while those born in 2003/04 lived only on average 699 days. The large drop seen at approximately 190 days in worst case survival outputs (Figure 4.5) corresponds to the large proportion of animals last seen prior to the subsequent season leaving the study as resighting only occurs during one period (summer season) per year. As a result, there is substantial uncertainty associated with estimating the temporal impacts of the variables on survival from these data.

Logistic regression for worst case outcome again was comparable to survival analysis with survival tending to be higher in 2002/03 compared to 2003/04 (OR 0.574, 95% CI 0.199-1.652; P=0.303). Sex had little impact on outcome (OR 1.192; 95% CI 0.416-3.412; P=0.744) while ivermectin treatment provided a protective effect with those pups treated being 2.7 times more likely to survive the 8-9 years to the end of the study period (95% CI 0.912-8.105; P=0.073).

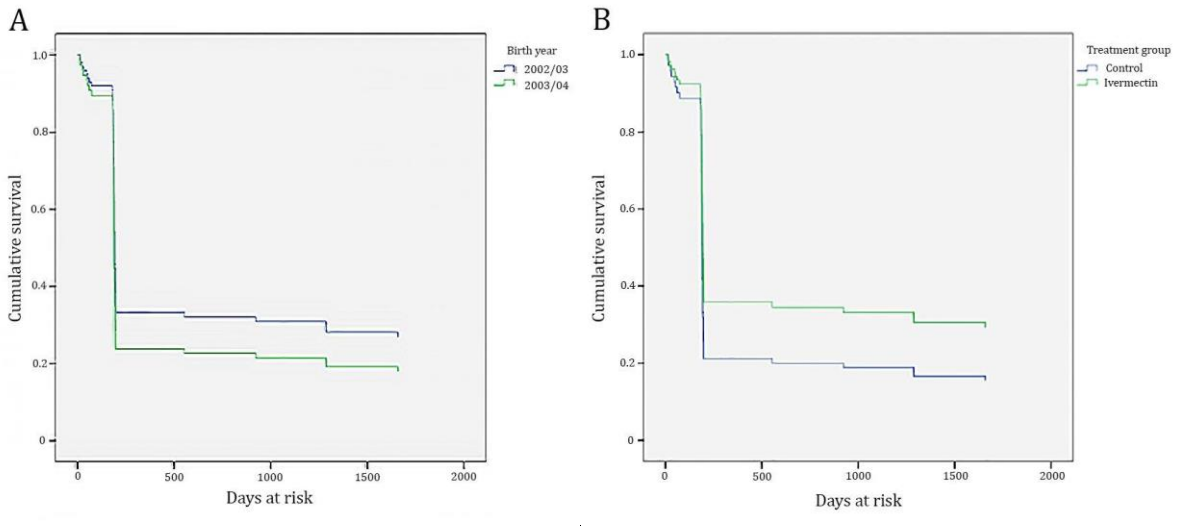

Figure 4.5 Worst case survival analysis using a Cox proportional hazards model for New Zealand sea lion (*Phocarctos hookeri*) pups with covariates A. birth year and B. ivermectin treatment group.

The average reproductive rate was 0.67. There was no significant difference in the reproductive rates of females born in 2002/03 (0.75, n=6) compared to 2003/04 (0.55, n=4; t=2.365, P=0.15) or those that were controls (0.83, n=3) compared to treated with ivermectin as pups (0.6, n=7; t=3.182, P=0.29). However, only ten females in the study (24%) reached the earliest pupping age of four years and all ten females were recorded as having pupped at least once.

4.5. Discussion

Long term fitness and reproductive outcomes have been shown to be significantly affected by early developmental conditions such as nutrition and disease (Metcalfe and Monaghan, 2001). Manipulation of these factors by administration of anthelmintics to remove worm burdens and therefore minimise nutritional losses by parasites and susceptibility to concurrent disease processes may aid in more than just neonatal survival. In addition to increased recruitment into the reproductive population following enhanced early survival, treated animals may then exhibit superior phenotypic quality and breeding success, with benefits extending subsequently to their offspring (Lummaa and Clutton-Brock, 2002). To our knowledge this is the first report of the effects of early removal of parasitic burden on long term survival and recruitment in a wild pinniped. The trend in the current data showing increased survival amongst the ivermectin treated pups is expected according to life history theory although over this extended time course there are innumerable potential contributing and confounding factors, many of which cannot be quantitatively assessed.

Small sample size is a common limitation for wildlife researchers, especially with already restricted and declining populations such as the NZ sea lion. However it should be recognised that the sample included here accounts for almost 9% of the total pup production in each year at Sandy Bay. Over 71% (*n*=60) of the sample either was confirmed to have died as a pup or was never resighted subsequent to the season in which they were born, drastically reducing the available sample size for longer term survival evaluation. Low estimated juvenile survival has previously been reported by Roberts and colleagues (2013) and is reflected in this sample but may also be compounded by the expected low resightability of juveniles and non-breeders (Chilvers and Wilkinson, 2008) as well as tag loss. Tag loss is an important confounding factor in any long-term study based on only resighting tags. Individuals that have lost both flipper tags and are not identifiable by other means such as branding or PIT (passive integrated transponder) tag are effectively indistinguishable from dead

animals, resulting in underestimation of survival. Additionally, the tags in some animals become worn, damaged or unreadable, again rendering these individuals effectively invisible for resighters. Although it is currently protocol to implant PIT tags into NZ sea lion pups during the tagging procedure, the pups in this study were tagged only, hence falling into this category as soon as both tags have failed. In NZ sea lions, the probability of losing one tag (from two tags to one, or one tag to zero) in the years following tagging has been estimated as 5-14% (Chilvers and MacKenzie, 2010, Roberts et al., 2013). These tag loss rates are within the reported range for otariids, but are readily impacted by variables such as type of tag, experience of tagger and age of animal when first tagged (Shaughnessy, 1994, Boyd et al., 1995). Additionally there are known sex specific differences, with male NZ sea lions being significantly more likely than females to lose both tags in the years after tagging (Chilvers and MacKenzie, 2010). This source of uncertainty could be ameliorated by ensuring animals are deployed with more than one type of identification, including more permanent methods such as PIT tags.

There was no apparent effect of ivermectin treatment on reproductive rate seen in this analysis however sample size was again a limiting factor. The average reproductive rate of 0.67 obtained from the ten females that were identified to reach maturity is consistent with previous reports (Childerhouse et al., 2010, Roberts et al., 2013). Additionally, this experiment could be improved by using an extended study period as these animals have only just entered the prime breeding age range of 7-12 years (Roberts et al., 2013).

Treatment of infectious disease in free-living wild populations of animals is often thought to be practically difficult and ineffective, although success is problematic to accurately measure (Woodroffe, 1999). Additionally, in this study, there was the confounding effect of bacterial disease affecting early survival of pups. Current research suggests that cryptic presentation of *K. pneumoniae* meningitis (affected pups may only exhibit subdural cerebellar haemorrhage) may be the true primary cause of death in pups diagnosed otherwise (Roe, 2012). As such this is an

important consideration in pups previously diagnosed as having hookworm enteritis based on gross post mortem alone. It is also unclear, that in the face of epizootic events whether removing a hookworm burden will save a pup from dying from bacterial infection. In the 2002/03 season in which there was an epizootic caused by *K. pneumoniae* resulting in over 21% pup mortality over three months, significantly more pups from the control group died compared to the ivermectin treated group (Chilvers et al., 2009). All pups had signs of *K. pneumoniae* infection on gross post mortem (Castinel et al., 2007b, Chilvers et al., 2009). Although three of these untreated pups were diagnosed with hookworm enteritis as a primary cause of death, it is much more likely that bacterial infection was the most life threatening process in these pups. On the other hand, the survival rate was significantly higher for treated compared to untreated pups in the epizootic year only (Chilvers et al., 2009), so there potentially is some benefit to pups in increasing available immune capabilities and improving general condition and blood volume in these conditions.

In addition to external factors, genetic predisposition has also been implicated in the propensity for NZ sea lion pups to succumb to hookworm infection, regardless of burden intensity (Acevedo-Whitehouse et al., 2009). The link identified between inbreeding and hookworm–associated mortality in California sea lion pups was not seen in NZ sea lions, further there was relatively high microsatellite allelic diversity amongst the Sandy Bay population (Acevedo-Whitehouse et al., 2006, Acevedo-Whitehouse et al., 2009). On the other hand, homozygosity at a particular microsatellite locus was associated with a strong predisposition to anaemia in both species, with a certain genotype being associated with significantly lower platelet counts (Acevedo-Whitehouse et al., 2009). Such pups would benefit from early clearing of intestinal parasites and prevention of worm attachment but selection in favour of pups with these anaemia-susceptible genotypes may result in the need for ongoing provision of anthelmintics.

Anthelmintic treatment has the greatest benefits for improving growth rate and survival in populations that have high intensity of parasites and high levels of hookworm-associated mortality.

In northern fur seals at San Miguel Island, both of these criteria are met. Administration of a single dose of ivermectin to northern fur seal pups resulted in significantly decreased (but not eliminated) hookworm burdens and improved growth and survival rates compared to controls (DeLong et al., 2009). On the other hand, inconsistent results were published following a study using praziquantel to treat tapeworm burden in Hawaiian monk seals (*Monachus schauinslandi*; Gobush et al., 2011). Aside from the demonstrated poor efficacy of praziquantel in removing adult cestodes in the study animals, the combined effect of a very small sample size and use of yearlings and juveniles only, it is difficult to draw comparison to the present study in NZ sea lions.

Avermectins are primarily eliminated unchanged by the faecal route and residues present in faeces of treated pups have the potential to affect various trophic levels of non-target organisms of the pristine sub-Antarctic ecosystem (Halley et al., 1989, Liebig et al., 2010). Ivermectin residues are well documented to have detrimental toxic effects on soil and aquatic organisms, particularly dung feeding flora and aquatic sediment invertebrates (Wall and Strong, 1987, Sanderson et al., 2007). In soil, ivermectin is persistent but readily degrades in light, hence reducing the available active drug in surface layers (Halley et al., 1993). In water, ivermectin is poorly soluble, the drug preferentially binding to sediment, with the potential for bioaccumulation at the benthos and in filter feeders in low concentrations (Davies et al., 1997). Brown skuas (*Catharacta antarctica*), common at sub-Antarctic NZ sea lion colonies commonly ingest pup faeces shortly after it is produced (S Michael, pers. obs.), hence potentiating the impacts of the drug. Although ivermectin is a relatively safe agent in birds, the widespread dissemination of the active ivermectin compound could impact negatively on parasite resistance over time and have toxic effects on susceptible organisms, particularly invertebrates, further afield from where initially dispensed. Although such environmental effects also exist where treated domestic animals are present, further work is required to fully understand the potential impacts in unique NZ sub-Antarctic conditions.

Our survival analysis has shown that influences of disease and parasitism on NZ sea lion pups during their first three months of life have wide ranging implications for long term survival, recruitment and reproductive success. Consequently, an improved understanding of the health and causes of mortality of pups in this early period is crucial for effective conservation management of this threatened species.

Chapter Five. General Discussion

The aim of this thesis was to examine several aspects of NZ sea lion reproductive success and evaluate whether infectious disease plays a role in the ongoing population decline of the species. Specifically, I assessed the importance of infectious agents in the aetiology of stillbirth and investigated the contribution of *Toxoplasma gondii* and hookworm (*Uncinaria* spp.) infection to fulfil the following aims:

1. Quantify stillbirth as a cause of NZ sea lion reproductive failure at Sandy Bay, Enderby Island and identify possible impacts of infectious disease from archived necropsy records and tissues
2. Determine the level of exposure to *T. gondii* amongst NZ sea lions from Sandy Bay, Enderby Island compared to Otago Peninsula and Stewart Island and identify potential impacts of the pathogen on reproductive success
3. Investigate long term survival and reproductive success of NZ sea lions that were treated with an anthelmintic as pups to remove hookworm burden and compare to a control group

5.1. Summary of results

Although stillbirth was found to account for a larger mean percentage of pup mortality (6%) at Sandy Bay than previously thought (4.2%; Castinel et al., 2007b), the rate still falls within the ranges reported in other otariid species (Table 5.1). Amongst the available sample of stillborn NZ sea lion pup tissues (n=37) and placentas (n=10), no evidence of infectious agents was identified. Inflammatory lesions were rare on histopathology, with neutrophilic or histiocytic pneumonia identified in four pups and no other lesions in other organs noted. Further investigation into the cause was not possible as frozen tissue had not been archived from these cases.

An unexpected finding of this work was the high incidence (86%; 31/36) of aspirated squames in the respiratory tracts of stillborn NZ sea lion pups. Generally this finding is associated with meconium aspiration as a consequence of foetal distress or hypoxia *in utero* (Hoffman Jr et al., 1974). No

meconium was identified in the airways of the sample of pups examined, so the significance is currently unclear. All placental tissue examined appeared normal on histopathology, consistent with normal births of apparently healthy pups. Although to date collection of placentas and tissues from stillborn pups has been sporadic, this study forms a baseline that can be elaborated in future seasons to form a wider view of late gestational reproductive failure in the species.

Table 5.1 Summary of pup mortality and stillbirth rates reported for otariids.

Species	Site and period	Mean pup mortality (%)[1]	Pup mortality range (%)	Mean stillbirth rate (%)[2]	References
Antarctic fur seal *Arctocephalus gazella*	Bird Island, South Georgia 1989-2003	22.4	7.2-47.4	7	(Reid and Forcada, 2005)
Australian sea lion *Neophoca cinerea*	Seal Bay, Kangaroo Island, Australia 2002-2006	30	24-34	7.6	(McIntosh and Kennedy, 2013)
New Zealand sea lion *Phocarctos hookeri*	Sandy Bay, Enderby Island, NZ 1997-2014	16.7*	6.4-52.6	6	(Castinel et al., 2007b, Roe, 2009, Childerhouse et al., 2014, unpublished data)
Northern fur seal *Callorhinus ursinus*	St Paul Island, Alaska, USA 2004	3.3	1.6-4.6	9**	(Towell et al., 2006, Spraker and Lander, 2010)
South American fur seal *Arctocephalus australis gracilis*	Guafo Island, Chile 2004-2008	6	4.2-9.2	2.6	(Seguel et al., 2013)
South American sea lion *Otaria flavescens*	Ballestas Islands, Peru 1997-2012	33.1	11.4-100	2.3	(Soto et al., 2004)

[1]Percentage of pup production that died during the monitored portion of the period described
[2]Percentage of total mortality that comprised stillbirths
*Pup mortality rate includes 'epizootic' and 'non-epizootic' years; 2012/13 season excluded due to lack of mortality data
**Mean stillbirth rate for the period 1986-2006

T. gondii seropositivity was confirmed in three mainland adult females (two at Otago Peninsula and one at Stewart Island), however all Enderby Island animals showed no evidence of exposure. *T. gondii* is known to affect most stages of the reproductive cycle with the ability to cause infertility, gestational failure, congenital birth defects and behavioural changes resulting in impaired survival (Dubey, 2010). In the two Otago females, there were no observed clinical effects of *T. gondii* and both were recorded to have produced healthy pups before and after the positive samples. These

findings are consistent with the comparatively high density of feral cats present on the mainland including Stewart Island (Moller and Alterio, 1999, Harper, 2005). Although feral cats are known to be present on Auckland Island (less than 10km from Enderby and Dundas Islands; Harper, 2010), the prevalence of *T. gondii* carriage in this group is not known. The findings of this study show that *T. gondii* infection is an unlikely contributor to the poor reproductive success of sub-Antarctic NZ sea lions.

Although the Cox proportional hazards model used in the analysis of ivermectin trial results and subsequent resighting records was unavoidably limited by small sample size, the study showed trends consistent with previous reports including improved early survival for anthelmintic treated pups (Chilvers et al., 2009, DeLong et al., 2009). Additionally, although not reaching statistical significance, there was a trend of improved long term survival for ivermectin-treated pups within the 8-9 year time period evaluated here. There were no differences in reproductive success between the groups, but the mean reproductive rate of 67% in the ten surviving females matches that reported by Childerhouse et al. (2010). A striking finding of the survival analysis was the poor juvenile survival beyond one year of age, although this result may have been influenced by small sample size and poor resightability due to dispersal and tag loss (Chilvers and MacKenzie, 2010). Poor juvenile survival markedly reduces the number of animals available for breeding and producing future generations of pups. Juvenile survival (to one year of age) has previously been estimated in NZ sea lions at between 30-70% (Chilvers and MacKenzie, 2010, Roberts et al., 2013), a lower rate than reported in other otariid species including California sea lions (55.6-99.8%; Hernández-Camacho et al., 2008b), NZ fur seals (≥85%; Bradshaw et al., 2003) and subantarctic fur seals (96.4%; Beauplet et al., 2005). Poor juvenile survival during the period of the current study may have been influenced by increased pup mortality due to *K. pneumoniae* infection but further work is required to assess the primary mediators. This analysis has shown that disease and parasitic effects on pups have the potential to have wide ranging implications on future fitness and survival, so management of these factors could be an important tool for strengthening population growth and capability.

5.2. The role of infectious diseases in New Zealand sea lion reproductive success

The overall dearth of identified infectious drivers to reproductive failure prior to birth is in contrast to the known impacts of infectious disease, particularly *K. pneumoniae*, on pups in the weeks following birth. Currently this pathogen is the primary cause of neonatal pup mortality at Enderby Island (Childerhouse et al., 2014). Given the continuing effects of the pathogen at this site (Roe, unpublished data) and the expected long term impacts of disease on future fitness of those pups that survive infection (Chapter Four), the drivers of neonatal morbidity and mortality require urgent attention for current and future conservation benefit.

Diseases known to cause reproductive failure in pinnipeds worldwide, including brucellosis, leptospirosis, coxiellosis, chlamydiosis, mycoplasmosis and toxoplasmosis (Burek et al., 2003, Lynch et al., 2011a, Lynch et al., 2011c, Minor et al., 2013) were not diagnosed amongst the sample tested. These agents generally exhibit characteristic inflammatory lesions on histopathology, which were not identified in the NZ sea lions. Furthermore, *Leptospira* spp. and *Brucella* spp. seroprevalence of NZ sea lions from mainland and sub-Antarctic sites has been previously been reported to be very low to absent (Roe et al., 2010), and *C. burnetii* is not known to be present in New Zealand (Anonymous, 2010). *T. gondii* causes high levels of mortality in southern sea otters in California, USA (Kreuder et al., 2003) and in Hector's dolphins in coastal NZ waters (Roe et al., 2013). NZ sea lions on the mainland share a range with Hector's dolphins on the southeastern coast of the South Island and based on work by Roe et al (2013), 2/3 dolphins necropsied from this region were PCR positive for *T. gondii*, with one of these diagnosed with fatal disseminated toxoplasmosis. It is unclear why only 2/10 NZ sea lions from this area were seropositive but may be a reflection of individual behaviour influencing exposure, such as inshore coastal foraging or increased time spent ashore near freshwater outflow channels.

The identified trends of improved early and long term survival of pups treated with ivermectin raises the question of the true impact of a hookworm burden on neonatal NZ sea lions. Survival following

deworming has been investigated in northern fur seal pups and juvenile Hawaiian monk seals, both with positive effects (DeLong et al., 2009, Gobush et al., 2011). The effect of increased growth rate by removing parasitic burdens has been well documented in domestic species, but improved survival, particularly under the influence of bacterial disease mediators such as *K. pneumoniae* is important and should be considered further as a potential mitigation tool to reduce pup mortality. The effects of anthelmintic treatment on pinnipeds beyond the season in which it was administered have not previously been reported and the results in NZ sea lions offer promising avenues for further assessment of its role in improving fitness and reproductive success in the long term. Concurrently, the effects of ivermectin on the environment and non-target hosts, particularly invertebrates, should be examined.

5.3. Conservation, management implications and future directions

This study has shown that the NZ sub-Antarctic islands remain relatively pristine with respect to pathogens known to be commonly present on the mainland. On the other hand, reproductive success, particularly early pup survival at this location is under the influence of infectious diseases that are not an identified problem on the mainland including *K. pneumoniae* and *Uncinaria* spp. (hookworm). These pathogens may contribute to early pup mortality and those pups that survive may be negatively affected into adulthood, however both infections may be amenable to conservation management intervention.

The pilot investigation into stillborn pups relied heavily on histopathology and identification of inflammatory lesions in archived tissues as a method of screening for multiple pathogens. In the future, molecular testing would be useful for identifying specific pathogens. Molecular techniques would complement histological screening in testing placental tissue for pathogens such as *C. burnetii* as PCR is significantly more sensitive than identification of gross and histologic lesions of placentitis (Duncan et al., 2012). In combination with further work in defining the role of disease in stillbirth,

additional field protocols are recommended, including collection of frozen and formalin-fixed placental tissue from stillborn pups to allow more extensive analysis at a later date. These measures will maximise the chances of reaching a diagnosis in cases of late gestational and peripartum mortality and improve our understanding of the factors involved in pup mortality. Further, examination of hormonal profiles of adult female NZ sea lions with collection of appropriately timed serum samples would be important to investigate pregnancy rates and the presence and magnitude of early embryonic loss. Progesterone measurement in serial blood samples has been reported as an accurate technique to estimate pregnancy rates and identify rates of embryonic loss in South African fur seals (*Arctocephalus pusillus*; Guinet et al., 1998) and NZ fur seals (McKenzie et al., 2005), however pregnant and non-pregnant animals cannot be distinguished until after implantation of the embryo after diapause. In NZ sea lions this is estimated to be approximately June, during which time sampling is practically difficult due to limited access and poor weather conditions at remote sub-Antarctic colonies and dispersal of females for foraging.

The finding of strong and persistent *T. gondii* seropositivity in several NZ sea lion females at Otago Peninsula and Stewart Island warrants surveillance for clinical effects and opportunistic retesting of serum where possible. The work presented in this thesis shows that both tests evaluated (LAT and ELISA), but particularly the LAT produced reliable results and could be used for surveillance in NZ sea lions, with further validation of the test as sample size increases.

Although parenteral ivermectin administration has been the most widely used in pinnipeds, other routes should be considered to minimise pup handling, therefore reducing the risk of stress and injury to animals. Ivermectin has been used frequently and effectively in domestic species to treat internal and external parasites as a topical pour-on preparation (Bisset et al., 1990, Pagé et al., 2000) with standard absorption and efficacy in all weather conditions that may be encountered in the sub-Antarctic, including rain immediately after application (Rolfe et al., 1997). Other endectocides such as selamectin are commonly used topically on domestic small animals of comparable size and weight

to NZ sea lion pups and have been shown in dogs and cats to be highly effective at removing internal and external parasite burdens with a wide safety margin (Bishop et al., 2000) while avoiding potential complications associated with injection of anti-parasitic drugs. Aside from its effective use for heartworm prophylaxis in red pandas in China (Lan et al., 2012), no reports have been published using selamectin in non-domestic animal species and any use in NZ sea lions would be off-label, however with research into its effects and pharmacokinetics it could provide a simple, effective and safe route of medicating pups to remove hookworm burdens. Efficacious removal of hookworm burdens in pups could also be achieved by treatment of pregnant adult females to break the life cycle before the lactogenic transmission phase. Complete removal of canine hookworm (*Ancylostoma caninum*) infection of domestic dog pups (also transmitted by the transmammary route) has been demonstrated by treatment of pregnant bitches with a single dose macrocyclic lactone (eg. doramectin, moxidectin; Schnieder et al., 1994, Epe et al., 1999). This may be a promising option for the treatment of sea lion pups. A large trial is recommended to further evaluate the benefits of worm burden removal in NZ sea lion neonates and assess environmental impacts beyond the intended host, particularly on invertebrates.

Overall the findings presented in this thesis show no evidence for infectious disease as a major impact on gestational and peripartum reproductive success. In contrast, some data presented particularly in Chapter Four has alluded to the influence of *K. pneumoniae* and *Uncinaria* spp. on pup and juvenile mortality, an area which should be carefully examined to understand pathogen dynamics in order to implement mitigation attempts. Planned work to address this knowledge gap involves a case control trial to identify risk factors involved in pup mortality including environmental factors (weather, substrate, habitat type), maternal factors (parity, age), pup factors (sex, age, external wounds, *K. pneumoniae* infection) and management factors (tagging, ivermectin treatment). Results from this study will direct future mitigation effort toward the strongest drivers of mortality.

5.4. Conclusions

- The role of infectious disease in stillbirth and late gestational failure is likely to be minimal, however bacterial disease caused by *K. pneumoniae* and parasitism by *Uncinara* spp. may be significant mediators of early pup survival.
- Disease and parasitism of NZ sea lion neonates may have long ranging negative effects on survival and reproductive success, which may contribute to ongoing population decline of the species.

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Chapter Seven. Appendices

Appendix 1. Grading criteria for histopathology of stillborn New Zealand sea lion tissues

Grade	Severity	Criteria
Tissue autolysis scale		
0	Not present	No autolysis
1	Mild	Reasonable preservation, early degradation
2	Moderate	Patchy regions of autolysis with areas of reasonable preservation
3	Marked	Large areas to complete autolysis, unable to define cell types or lumen of airways
Squame aspiration scale		
0	Not present	Not present
1	Mild	Occasionally seen
2	Moderate	Seen frequently, but not every high power field, includes cases with focal severe accumulations, occasionally present within cartilaginous airways
3	Marked	Seen commonly, on most high power fields, present within most cartilaginous airways
Liver vacuolation scale		
0	Not present	No vacuolation, resembles an adult liver
1	Mild	Resembles adult liver but with small vacuoles present in hepatocytes on high power
2	Moderate	Medium sized vacuoles present in most hepatocytes, vacuolation evident diffusely from low power
3	Marked	Large vacuoles obscure structure of most hepatocytes, vacuolation obvious and marked on low power

Appendix 2. Histopathology grades of lung and liver tissue from stillborn New Zealand sea lions

Enderby Case Number	Histology Case Number	Pup Sex	Tissue Autolysis	Atelectasis	Eosinophilic fluid in airways	Squames in airways	Alveolar macrophages	Giant cells	Neutrophils in airways	Meconium in lungs	Liver vacuolation	Comments
E99/00-33Ph	E362-12	M	1	2	1	1	1	0	0	0	1	One lung section worse than the other
E00/01-3Ph	E363-12	M	1	3	0	1	2	1	1	0	2	
E00/01-8Ph	E364-12	F	0	3	1	2	2	0	1	0	0	Focal severe squame accumulations, other areas absent
E01/02-2Ph	E365-12	F	2	3	0	3	3	2	1	0	0	Mainly cells in airways, large giant cells
E01/02-5Ph	E366-12	M	2	3	2	1	0	0	0	0	1	
E01/02-14Ph	E367-12	M	1	3	2	1	0	0	0	0	2	Fluid in large airways
E01/02-18Ph	E368-12	M	2	3	3	3	0	0	0	0	2	PAS negative
E02/03-4Ph	E369-12	F	0	2	0	3	1	0	0	0	3	Squames in large airways
E02/03-5Ph	E370-12	F	2	3	3	2	1	0	0	0	2	Fluid and squames in large airways
E03/04-26Ph	E371-12	M	1	2	0	1	0	0	0	0	3	
E04/05-2Ph	E141-05	M	2	3	2	2	0	0	0	0	-	PAS negative
E04/05-6Ph	E136-05	F	3	3	3	1	1	a	a	a	-	Fluid in large airways
E04/05-8Ph	E133-05	F	-	-	-	-	-	-	-	-	3	
E04/05-12Ph	E125-05	F	2	2	2	1	2	0	1	0	1	
E04/05-49Ph	E137-05	M	1	2	2	0	0	0	0	0	2	Partial aeration of lung in one section
E05/06-6Ph	E372-12	F	2	3	3	2	2	0	0	0	-	Hard to determine cell types
E05/06-7Ph	E373-12	M	0	2	2	0	1	0	0	0	3	
E06/07-3Ph	E374-12	F	1	2	2	2	0	0	0	0	1	Partial aeration of lung, squames in large airways
E06/07-5Ph	E375-12	M	2	3	3	0	1	0	0	0	1	

- tissue not available for histopathology
a tissue too autolysed to determine grading on histopathology

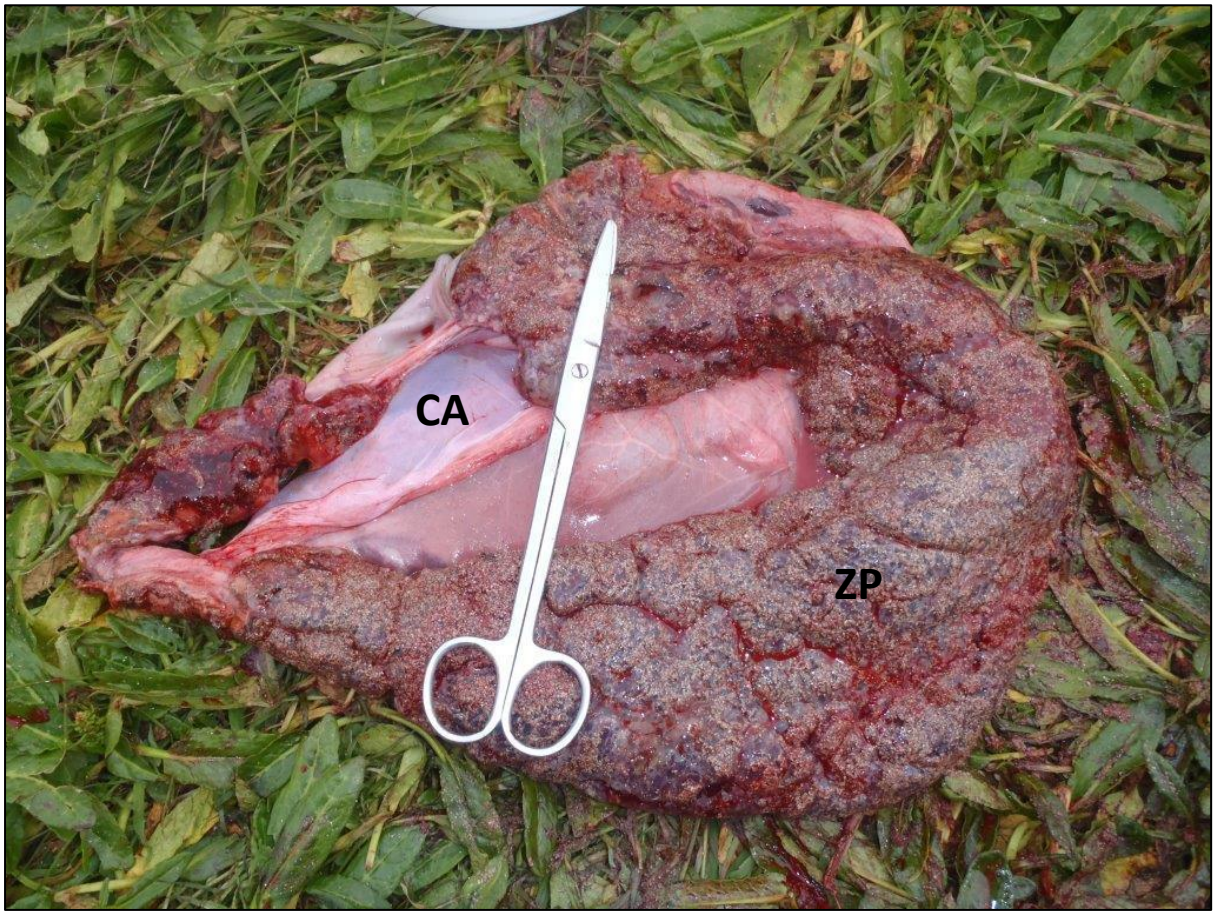
Appendix 2. Histopathology grades of lung and liver tissue from stillborn New Zealand sea lions continued

Enderby Case Number	Histology Case Number	Pup Sex	Tissue Autolysis	Atelectasis	Eosinophilic fluid in airways	Squames in airways	Alveolar macrophages	Giant cells	Neutrophils in airways	Meconium in lungs	Liver vacuolation	Comments
E06/07-8Ph	E376-12	M	1	2	0	2	0	0	0	0	1	Sloughed epithelium and squames in large airways
E06/07-10Ph	E377-12	M	0	2	1	0	0	0	0	0	1	
E06/07-24Ph	E378-12	F	2	3	1	2	1	0	0	0	-	
E08/09-2Ph	E379-12	M	3	2	a	2	a	a	a	a	0	Post mortem bacterial invasion with Gram positive rod
E08/09-16Ph	E381-12	F	0	2	1	1	1	0	0	0	3	Focal severe squame accumulations, other areas absent
E08/09-17Ph	E382-12	F	1	2	1	3	1	0	0	0	3	Squames in large airways
E09/10-1Ph	E383-12	M	0	3	1	3	0	0	0	0	0	PAS negative
E09/10-2Ph	E384-12	M	3	3	2	2	a	a	a	a	-	
E09/10-3Ph	E385-12	F	2	3	2	0	0	0	0	0	2	
E09/10-9Ph	E386-12	M	2	3	2	2	0	0	0	0	1	
E10/11-1Ph	E387-12	M	2	2	1	1	0	0	0	0	3	
E10/11-2Ph	E388-12	F	1	2	0	2	2	0	0	0	3	Focal severe squame accumulations, other areas absent
E10/11-3Ph	E389-12	F	0	2	1	2	2	0	0	0	2	
E10/11-4Ph	E390-12	M	0	1	0	1	2	0	0	0	2	Areas of relatively well inflated lung with squames within
E10/11-5Ph	E391-12	M	2	2	0	2	1	0	0	0	1	Focal severe squame accumulations, other areas absent
E10/11-6Ph	E392-12	F	3	3	2	2	a	a	a	a	2	
E10/11-10Ph	E393-12	M	2	2	2	2	1	0	0	0	-	
E11/12-14Ph	E394-12	F	0	1	0	1	0	0	0	0	3	

-; tissue not available for histopathology

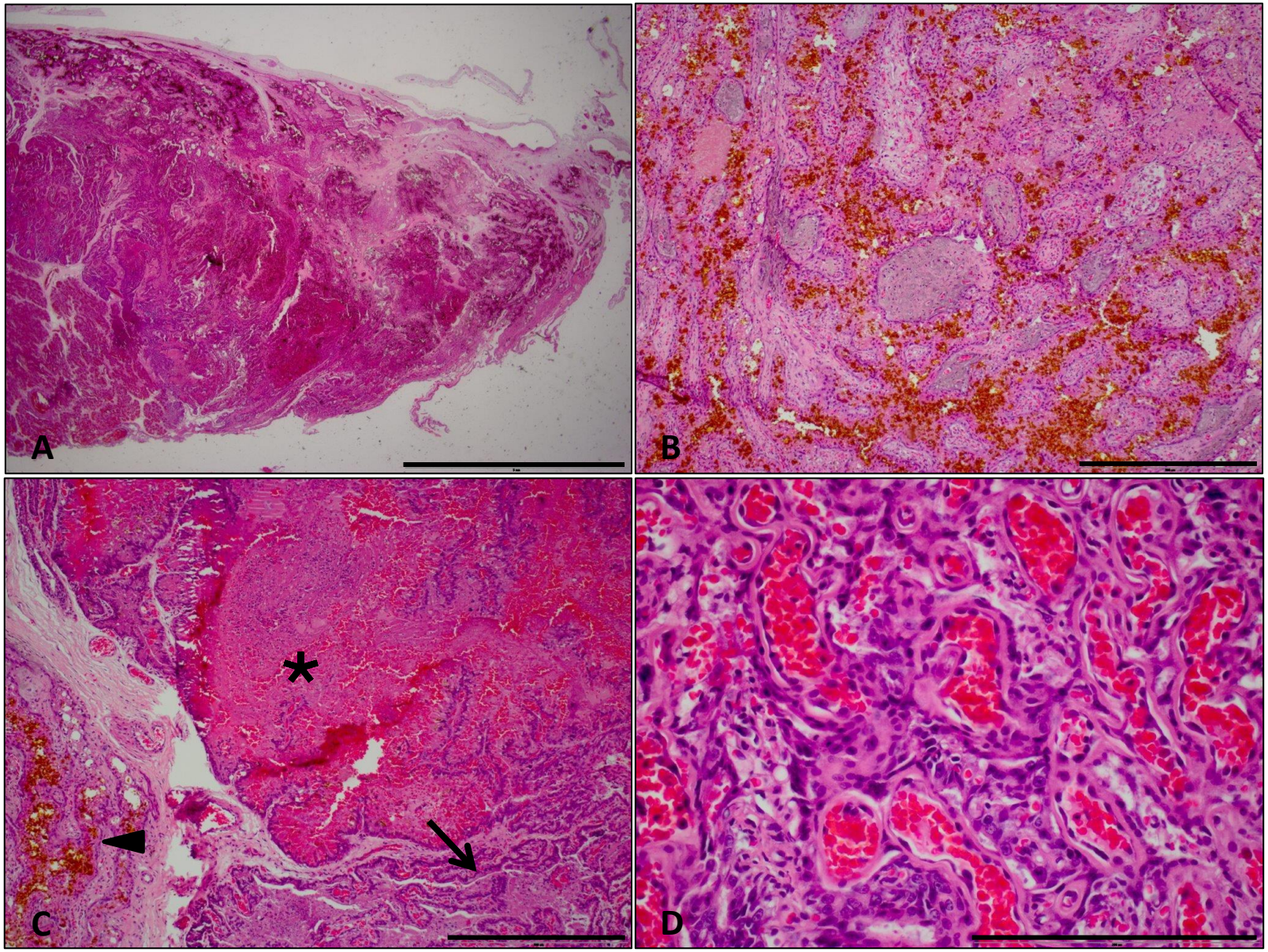
a; tissue too autolysed to determine grading on histopathology

Appendix 3. Normal New Zealand sea lion placenta



Appearance of normal zonary placenta (ZP) and chorioallantois (CA). Image credit: BL Chilvers.

Appendix 4. Histology of normal New Zealand sea lion placenta



Haematoxylin and eosin. A. Zonary placenta, scale bar = 5mm; B. Haemophagous zone with prominent haemosiderin deposits, scale bar = 500µm; C. Marginal haematoma (asterisk) adjacent haemophagous zone (arrow head) and chorionic villi (arrow), scale bar = 500µm; D. Chorionic villi, scale bar = 200µm

Appendix 5. SDS PAGE procedure for *Toxoplasma gondii* western blot

Protein concentration of the pre-prepared antigen (Toxovax, MSD Animal Health) was measured with spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE, USA). Several concentrations of antigen protein (10, 20 and 40µg) were mixed with equal volumes of 2x Laemmli buffer containing 65.8mM Tris-HCl (pH 6.8), 26.3% (w/v) glycerol, 2.1% SDS, 0.01% bromophenol blue and 10% 2-mercaptoethanol (Bio-Rad, Hercules, CA, USA) and denatured by heating at 100°C for 5 minutes. The denatured protein (48µg total antigen/gel) and a protein marker with a molecular weight range of 10-250kDa (Precision Plus Protein Dual Color Standards, Bio-Rad) were run through a 12.5% Tris-HCl gel (Criterion Prep+2, Bio-Rad) for 150 minutes at 120V in a Tris-glycine SDS buffer containing 25mM Tris, 192mM glycine and 0.1% (w/v) SDS (pH 8.3) (Bio-Rad). After separation the gel was removed and stained with Coomassie stain (1.25% Coomassie Blue dissolved in 250mL methanol with 200mL distilled deionised water and 50mL acetic acid) for 30 minutes then destainer (75% methanol, 25% acetic acid) at 4°C overnight until sufficiently destained. The gel was then visualised with an ultraviolet transilluminator.

Appendix 6. Presence of *Toxoplasma gondii* antibodies in sera collected from New Zealand sea lions at mainland and sub-Antarctic locations

ID	Sex	Capture location[a]	Year sampled	Age (years)[b]	LAT Titre	ELISA S/P Value (%)	Western Blot[c]
0350	F	OP	2008	14	2048	27.7	+
0350	F	OP	2010	16	2048	24.9	+
0351	F	OP	2008	12	0	-1.5	nt
2578	F	OP	2009	7	0	-1.5	nt
2578	F	OP	2010	8	0	-1.0	nt
2580	F	OP	2009	7	4096	20.5	+
2580	F	OP	2010	8	4096	19.1	+
2582	F	OP	2008	5	0	-3.4	nt
2582	F	OP	2010	7	0	-1.7	-
2584	F	OP	2008	4	0	-2.2	-
2587	F	OP	2009	4	0	-1.6	nt
2587	F	OP	2010	5	0	0.8	nt
2588	F	OP	2009	4	0	-3.3	nt
2591	F	OP	2009	3	0	-0.9	nt
3455	F	OP	2010	2	0	-1.6	nt
6064	F	SI	2013	8	0	-1.8	nt
E788	F	SI	2013	A	0	-1.1	nt
E789	F	SI	2012	A	0	-1.7	-
E790	F	SI	2012	A	0	-1.5	nt
E792	F	SI	2012	A	0	-1.7	nt
E793	F	SI	2012	A	0	-2.2	nt
E794	F	SI	2012	A	0	-1.9	nt
H823	F	SI	2013	A	0	-1.0	nt
H824	F	SI	2013	A	0	-0.9	nt
H825	F	SI	2013	A	4096	20.1	+
H826	F	SI	2013	A	0	-1.8	nt
H828	F	SI	2013	A	0	-1.1	nt

[a] OP, Otago Peninsula; SI,Stewart Island; EI, Enderby Island.

[b] Age denotes the animals age at the time of sampling. A denotes that the animal had been tagged as an adult so accurate aging was not possible.

[c] +, positive recognition of bands consistent with *T. gondii* on western blot; c, positive recognition of bands not consistent with *T. gondii* on western blot; -, no bands recognised on western blot; nt, not tested.

Appendix 6. Presence of *Toxoplasma gondii* antibodies in sera collected from New Zealand sea lions at mainland and sub-Antarctic locations continued

ID	Sex	Capture location[a]	Year sampled	Age (years)[b]	LAT Titre	ELISA S/P Value (%)	Western Blot[c]
0088	F	EI	2011	11	16	-1.0	nt
0117	F	EI	2011	11	16	0.0	nt
0119	F	EI	2011	11	0	-0.7	nt
0152	F	EI	2011	11	16	0.0	-
0240	F	EI	2012	12	16	-0.5	nt
0241	F	EI	2010	10	16	9.5	c
0375	F	EI	2011	11	0	-0.1	nt
0415	F	EI	2012	12	16	-0.3	nt
0972	F	EI	2009	A	32	-0.3	nt
1411	F	EI	2010	16	0	0.5	nt
1758	F	EI	2009	8	16	11.8	c
3857	F	EI	2011	8	0	0.3	nt
3873	F	EI	2011	8	0	-0.4	nt
3926	F	EI	2011	8	0	-0.6	nt
3939	F	EI	2011	8	0	-0.6	-
3978	F	EI	2011	8	0	-0.6	nt
4063	F	EI	2011	8	0	-0.7	nt
4775	F	EI	2011	7	16	-0.7	nt
4840	F	EI	2011	7	0	0.6	nt
5703	F	EI	2012	7	0	-0.4	nt
5752	M	EI	2010	5	16	-0.1	nt
5770	F	EI	2012	7	0	4.4	c
6021	F	EI	2012	7	32	0.3	c
6363	F	EI	2009	3	0	1.0	nt
7260	M	EI	2010	3	0	-0.4	nt
B0179	F	EI	2010	11	0	13.6	c
B0581	F	EI	2011	12	16	-0.4	nt
H227	F	EI	2010	1	0	0.2	nt

[a] OP, Otago Peninsula; SI,Stewart Island; EI, Enderby Island.

[b] Age denotes the animals age at the time of sampling. A denotes that the animal had been tagged as an adult so accurate aging was not possible.

[c] +, positive recognition of bands consistent with *T. gondii* on western blot; c, positive recognition of bands not consistent with *T. gondii* on western blot; -, no bands recognised on western blot; nt, not tested.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