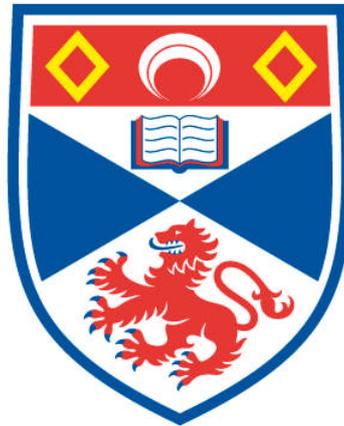


THE PATHOLOGY AND OCCURRENCE OF PATHOGENS IN SCOTTISH GREY SEALS (HALICHOERUS GRYPUS).

Johanna L. Baily

**A Thesis Submitted for the Degree of PhD
at the
University of St Andrews**



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**The pathology and occurrence of pathogens in
Scottish grey seals (*Halichoerus grypus*).**

Johanna L. Baily

A thesis submitted for the degree of Doctor of Philosophy

School of Biology, University of St Andrews

January 2014

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“The great mammals of the sea have much to tell us,

if only we learn to listen”

Michelle Lynn Reddy, Leslie A. Dierauf and Frances M. D. Gulland

Thesis abstract

Neonatal mortality in grey seals on the Isle of May breeding colony and in a rehabilitation centre were investigated by detailed systematic post-mortem examinations (n=59), on-site bacteriology and advanced molecular diagnostic techniques for specific pathogens. Causes of death on the breeding colony included starvation (30%), omphalitis-peritonitis (26%), septicaemia (22%), stillbirth (10%) and trauma (4%) and in the rehabilitation centre starvation (44%) and septicaemia (22%). Detailed key gross and histopathological findings and pathogens are described and include the first report of *Listeria monocytogenes* in any marine mammal. Phocid herpes virus 1 nucleic acids were detected in nasal swabs of 58% live, free-ranging grey seal pups (n=90) and 28% yearlings (n=19), suggesting recrudescence in the latter. Previously undetected in Scotland, phocid herpes virus 2 nucleic acids were identified only in yearlings (15%); sealpox was detected in a single live stranded grey seal pup and phocine distemper virus was not detected.

Given their unique characteristics and potential for acting as sentinels of coastal marine health several pathogens of putative anthropogenic origin were investigated: *Toxoplasma gondii*, *Neospora caninum*, *Salmonella spp.* and *Campylobacter spp.*. *Toxoplasma gondii* DNA was detected in 6% dead free-ranging grey seal pups (n=50) but *N. caninum* was not found.

Salmonella (20%) and *Campylobacter* (50%) were isolated from rectal swabs of live and dead grey seal pups and *Campylobacter* was significantly associated with moderate to severe colitis implying pathogenicity. These findings imply a land-sea-land transfer of *T. gondii* and early exposure of pups to this parasite. Extensive genetic fingerprinting suggested an exchange of *Salmonella* between grey seal, cattle and human populations and that the *Campylobacter* isolates may share the same origin as human clinical isolates.

This work provides a solid base line study of diseases present in grey seal pups and demonstrates that they are useful indicators of coastal marine microbial contamination.

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List of frequently cited marine mammal species

Pinnipeds

Grey seal	<i>Halichoerus grypus</i>
Harbour (common) seal	<i>Phoca vitulina</i>
Harp seal	<i>Pagophilus groenlandicus</i>
Hooded seal	<i>Cystophora cristata</i>
Ringed seal	<i>Pusa hispida</i>

Otariids and Odobenidae

Walrus	<i>Odobenus rosmarus</i>
Northern elephant seal	<i>Mirounga angustirostris</i>
Southern elephant seal	<i>Mirounga leonine</i>
Stellar sea lion	<i>Eumetopias jubatus</i>
California sea lion	<i>Zalophus californianus</i>
Antarctic fur seal	<i>Arctocephalus gazella</i>
New Zealand sea lions	<i>Phocarctos hookeri</i>

Other marine mammals

Killer whale	<i>Orcinus orca</i>
Harbour porpoise	<i>Phocoena phocoena</i>
Sea otter	<i>Enhydra lutris nereis</i>

List of frequently used abbreviations

Ab	Antibody
adk	Adenylate kinase
ANOVA	Analysis of variance
aspA	Aspartase A
atpA	ATP synthase alpha subunit
BIGSdb	Bacterial Isolate Genome Sequence Database
BLAST	Basic local alignment search tool
CCDA	Charcoal cefoperazone desoxycholate agar
CHV1	Canid herpesvirus 1
CO ₂	Carbon dioxide
CSBA	Columbia agar with sheep blood
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
FeHV1	Felid herpesvirus 1
FET	Fisher's exact test
GLM	Generalized linear model
glnA	Glutamine synthetase
gltA	Citrate synthase
glyA	Serine hydroxymethyltransferase
H&E	Haematoxylin and eosin
H ₂ S	Hydrogen sulfide
hipO	Hippurate hydrolase
HSD	Honestly significant difference
HT	High Throughput
IHC	Immunohistochemistry
LB	Luria-Bertani
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable number of tandem repeats analysis
MRI	Moredun Research Institute
NaOH	Sodium hydroxide
OR	Odds ratio
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDV	Phocine distemper virus
PFGE	Pulsed-field gel electrophoresis
pgi	Glucose-6-phosphate isomerase
pgm	Phosphoglucomutase
PhHV1	Phocid herpesvirus 1
PhHV2	Phocid herpesvirus 2

RNA	Ribonucleic acid
Rnase	Ribonuclease
rRNA	Ribosomal RNA
SAC	Scottish Agricultural College
SAC CVS	SAC Consulting Veterinary Services
SPCA	Society for the prevention of cruelty to animals
SRUC	Scotland's Rural College
ssDNA	Single stranded DNA
TAE	Tris acetate-EDTA
tkt	Transketolase
UK	United Kingdom
uncA	ATP synthase α subunit
UPGMA	Unweighted pair group method with arithmetic mean
UV	Ultra violet
VNTR	Variable number of tandem repeats
ZN	Ziehl-Neelson
95% CI	95% confidence interval

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Chapter 1 General introduction

1.1 Grey seals

Grey seals (*Halichoerus grypus*) belong to the suborder of pinnipeds, which means literally “fin-footed” in Latin in reference to their distinctive flippers. They are a group of mammals that are highly adapted to the aquatic, primarily marine, environment characterised by their streamlined shape, indistinct pinnae, large eyes and aforementioned flippers. This suborder contains the Phocidae (“true” or “earless” seals, including the grey seal) along with the Otariidae (fur seals and sea lions) and the Odobenidae (walrus (*Odobenus rosmarus*)) (Berta 2009). From a phylogenetic perspective pinnipeds are thought to be derived from a common bear-like ancestor in the Oligocene epoch, display many similarities to the present day bear (*Ursus* sp.) and domestic dog (*Canis canis*) and are classed in the Order Carnivora (Heyning & Lento 2002). They are elegantly adapted to life at the interface of the aquatic and terrestrial environment with numerous highly specialised cardio-vascular and pulmonary adaptations to diving and abundant insulating blubber (Rommel & Lowenstine 2001).

Grey seals inhabit the temperate and sub-arctic Western and Eastern shores of the North Atlantic forming distinct populations centred on the Baltic Sea, the eastern North Atlantic and western North Atlantic (Hall & Thompson 2009). Although they spend the large majority of their time in the water, they regularly haul out on land or ice throughout the year with prolonged haul-outs during the breeding season (September to November in the UK) and at their annual moult (January to March in the UK) (Reeves et al. 2002). Telemetry studies have demonstrated that grey seals predominantly forage in localized areas characterized by a gravel/sand seabed sediment within a relatively short distance from their haul out sites but also travel up to 2100km to distant haul out-sites (McConnell et al. 1999).

Chapter 1 – General Introduction

Female grey seals give birth to a single pup weighing, on average, 14kg which is covered in a long creamy white lanugo coat that is typically shed around the third week of life (Figure 1-1). Female seals (cows) provide immunoglobulin G (IgG) laden colostrum (Carter et al. 1990) and lipid rich milk enabling pups to triple their body weight within a few weeks of birth. Pups remain on shore until weaning at 3-4 weeks of age when they weigh, on average, 45-50kg. Subsequent to weaning they can either go to sea immediately or remain on land for up to a month, living off their blubber reserves, until they finally depart (Hewer 1964).



Figure 1-1 Grey seal cow and pup on the Isle of May colony

The average age of female recruitment into the breeding population varies between 5.5 to 9 years of age depending on the colony studied (Hammill & Gosselin 1995; Pomeroy et al. 2010). Sexual maturity occurs at an average of 5.6 years for males (Hammill & Gosselin 1995) with bulls on average 10 years of age before first breeding (Atkinson 1997). Mating occurs either on land or in the water shortly after the end of the lactation period (Atkinson 1997; Twiss et al.

2006). A blastocyst rapidly forms, which remains in embryonic diapause for approximately 100 days prior to uterine implantation. The placental gestation period is 8 months giving a total gestation period of 11 to 11.5 months (Atkinson 1997; Backhouse & Hewer 1956; Backhouse & Hewer 1964). In general, female grey seals display strong site fidelity, returning to the same colony to breed in successive years and both sexes demonstrate strong philopatry, most often breeding at their colony of birth (Pomeroy et al. 1994; Pomeroy et al. 2000b). Neither bulls nor cows feed during the pupping and mating seasons and consequently lose a substantial amount of body weight at this time (Pomeroy et al. 1999).

1.2 Scottish grey seals

Monitoring of grey seals is based on numbers of pups, obtained through repeated aerial surveys (Sea Mammal Research Unit 2012). Estimating the size of the entire population is more challenging, requiring the use of complex models which integrate estimates of age-specific fecundity rates, both pup and non-pup survival rates (Lonergan et al. 2011b; Sea Mammal Research Unit 2012) and incorporation of telemetry data (Lonergan et al. 2011a). In 2012, the British grey seal population was estimated at 111,300, representing approximately 38% of the world's population of grey seals. Almost 90% of these animals breed in Scotland, on remote beaches or islands, with the main concentrations in colonies in the Outer Hebrides, Orkney and the Farne Islands (Sea Mammal Research Unit 2012). In the UK, the pupping season begins in August and September off the Scilly Isles and Cornwall and becomes progressively later in a clockwise fashion around the British Isles, with the last pups being born on the East coast of Scotland and the Farne Islands in late December (Sea Mammal Research Unit 2012).

- **Sympatric pinniped species**

Only one other species of pinniped is resident along the British coastline: the Eastern Atlantic harbour (or common) seal (*Phoca vitulina*) the population of which is currently estimated at 36,500 (Sea Mammal Research Unit 2012). This species is significantly smaller than the grey seal but occupies markedly overlapping habitats at a geographical scale. Harbour seals tend to haul out on sandy and pebble beaches, intertidal rocks and ledges, and sandbars (Reeves et al. 2002) where they breed during the summer months (June/ July). They give birth to a precocious pup, often below the high water mark, which is able to swim and dive minutes after birth (Burns 2009).

1.2.1 Conservation status and Legislation

Although grey seals are classified as “least concern” by the International Union for Conservation of Nature (Thompson & Härkönen 2008), the Marine (Scotland) Act was passed on the 10th March 2010 superseding the Conservation of Seals Act 1970. The former provides improved protection of seals in Scotland by establishing a strict and comprehensive licence system to ensure appropriate management. It is an offence to kill or take any seal at any time throughout the year except under licence or for animal welfare concerns. This act allows for a degree of management to protect fisheries and fish farms (Scottish Parliament 2010). The Conservation of Seals Act is however, still extant in England.

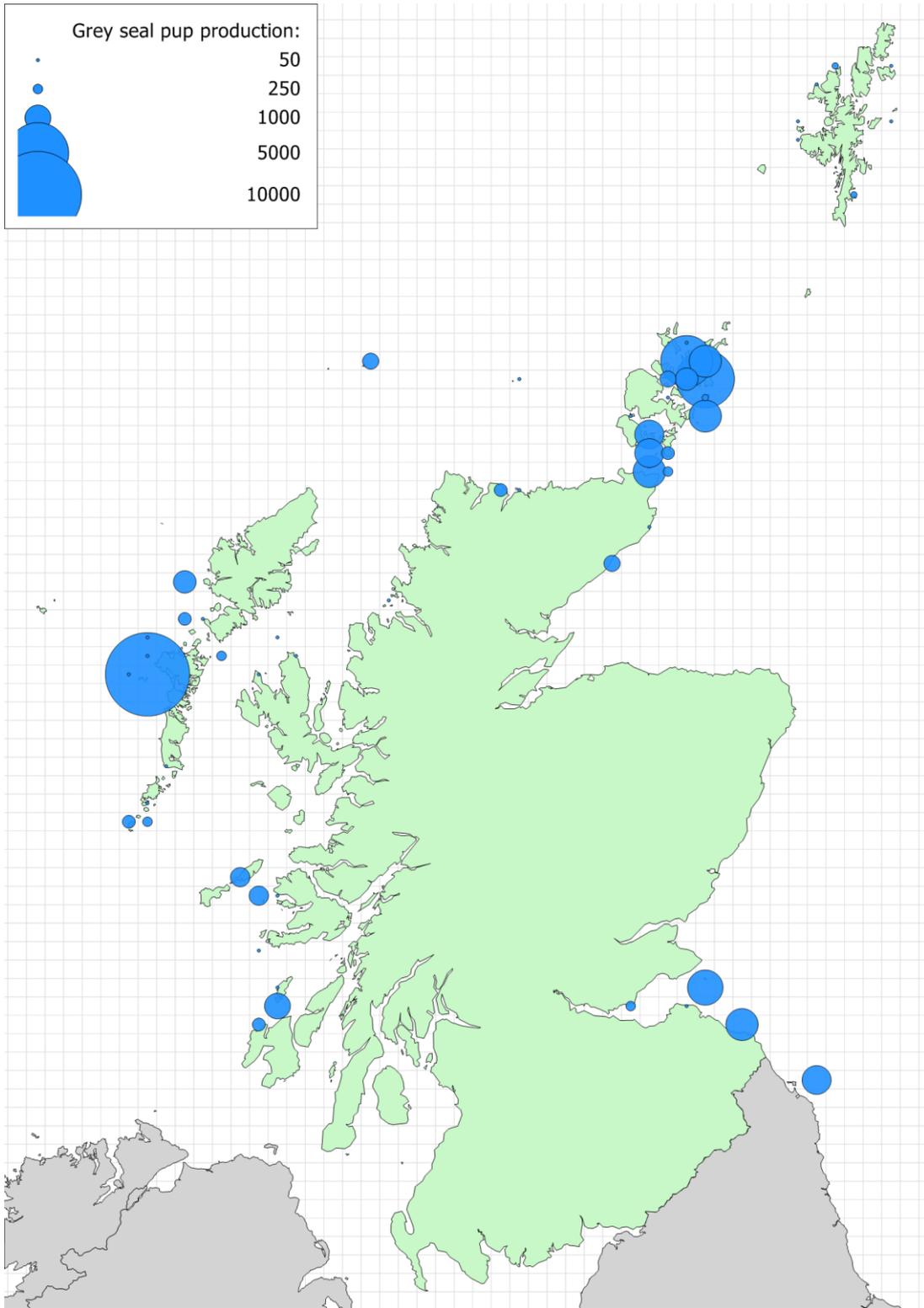


Figure 1-2 Grey seal pup production estimates for Scottish breeding colonies in 2010 aggregated by 10km squares. Data were obtained in 2009 and 2010 by SMRU aerial surveys and SNH ground counts (in Shetland). Data are aggregated by 10km squares with size of each dot proportional to number of pups born annually and centred on each square. Map generated by Chris Morris, SMRU using Manifold GIS.

Grey seals are recognised as a key species of the UK marine environment and, as such, figure in the EU Marine Strategy Framework Directive (2008/56/EC) as a key predator species. This directive aims to achieve good environmental status in EU waters by 2020 by focusing on maintaining populations of key trophic groups or key species within recommended levels. First year survival is recognised as a key driver of many wild mammal populations (Sinclair 1996), therefore assessing the causes of neonatal mortality will help understand grey seal population fluctuations and also provide a baseline of “normal” attrition.

1.2.2 Population dynamics

Although the number of grey seal pups born throughout Britain has grown steadily since records began in the 1960s, grey seal populations around the North and North West coasts of Scotland, Orkney, North Rona and the Monach Islands now show clear evidence of reaching a plateau or entering a slow decline based on aerial counts and estimates of pup production (Sea Mammal Research Unit 2012). In contrast, pup production within the North Sea colonies has continued to increase exponentially (Fast Castle, Donna Nook, Blakeney Point and Horsey colonies) with the exception of the two well established colonies of the Isle of May and the Farne Islands where pup production is beginning to plateau (Sea Mammal Research Unit 2012). Differences in pup survival and adult fecundity have both been proposed as possible explanations for this variation between colonies (Newman et al. 2009) with a reduction in pup survival currently thought to be the key driver of the recent slowing of the rate of growth of the population (Lonergan et al. 2011a).

Pre- and post-weaning mortality rates of grey seal pups have been estimated for different Scottish grey seal colonies using a complex series of capture/recapture/resighting studies and correlated with features such as sex, body mass, condition and serum immunoglobulin levels (Hall et al. 2002). Increased body mass or condition at weaning was shown to have a positive

effect on the first year survival rates of grey seal pups born on the Isle of May and the Farne Islands (Hall et al. 2001; Hall et al. 2002). However, male pups had a significantly lower probability of post-weaning survival compared to female pups. On the Isle of May in 1998 the estimated overall annual survival rate for male grey seal pups from weaning to one year of age was 0.193 compared to 0.617 for females (Hall et al. 2001). Surprisingly, higher serum concentrations of immunoglobulin G (IgG) at weaning were associated with a decreased probability of post-weaning survival (Hall et al. 2001; Hall et al. 2002). The authors were unable to comment on the significance of this finding as it was unclear whether the pups with higher serum concentrations of IgG were individuals with naturally higher circulating concentrations or because titres were elevated due to antigenic challenge at the time of sampling, however molecular or genetic factors may be responsible for this difference (Hall et al. 2002). Studies in Canada agreed with these findings, showing that grey seal pup birth mass and mass at weaning positively correlated with maternal age and mass in a 23 year study in Sable Island, Nova Scotia (Bowen et al. 2006).

The population of harbour seals in Scottish coastal waters has seen a substantial (up to 85%) decline since the late 1990s. This is most marked in Orkney, Shetland and the East coast of Scotland, whereas populations of harbour seals on the North West coast of Scotland remain stable (Lonergan et al. 2007; Sea Mammal Research Unit 2012; Thompson et al. 2005). The cause of this decrease remains unknown but reduced food availability, interspecific competition between harbour seals and grey seals, predation, biotoxins, disease and other anthropogenic factors have all been proposed (Hall & Frame 2010; Hanson et al. 2013; Sea Mammal Research Unit 2012).

1.2.3 Threats to Scottish seal populations

Despite their roles as top marine predators in UK waters, grey and harbour seals face a number of threats. Trauma from several different sources is a commonly reported cause of death e.g. human interaction such as ship propellers, intra-species fighting, adverse weather conditions or entanglement in commercial fishing nets leading to by-catch mortality (Moore et al. 2009; Moore et al. 2013). Commercial nor subsistence harvesting of seals does not occur in the UK but controlled culls by fisheries and fish farms are legal under appropriate licensing, within the Marine (Scotland) Act, 2010 (Scottish Parliament 2010). In addition to trauma there is also the threat of direct human competition due to overfishing and therefore depletion of food sources. As a relatively timid species, grey seals seek barren rocky locations as breeding sites (Harwood 2001; Harwood & Wylie 1987). Consequently, increased human disturbance due to farming activity, tourism and energy generation projects may negatively impact on their reproductive success (Harwood 2001; Skeate et al. 2012).

Given their relative longevity (potential lifespan 35-40 years), position at the top of the food chain and abundant blubber reserves, seals are susceptible to bio-accumulation of lipid soluble pollutants such as polychlorinated biphenyls (PCBs), commonly used in capacitors and transformers, or, historically, the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT), both of which can accumulate in high concentrations in tissues such as the blubber, liver and brain (Jenssen 1996; Jenssen et al. 1996; Troisi et al. 2001). These pollutants have been shown to adversely affect immune parameters of several marine mammal species (Brouwer et al. 1989; Hall & Thomas 2007; Hammond et al. 2005a) and are likely to lead to increased susceptibility to disease (Bredhult et al. 2008; Troisi et al. 2001). Furthermore, higher blubber contaminants at weaning were found to be associated with decreased probability of first-year survival of grey seal pups when controlling for sex and condition at weaning (Hall et al. 2009).

Disease inevitably impacts on marine mammal populations, both by directly resulting in mortality and indirectly by decreasing reproductive capacity, immunosuppression or increased parasite burden (Bossart 2011). It is essential to study disease in these species with the entire ecosystem in mind, allowing for the consideration of multiple factors such as stress, nutrition and pollution along with the standard host-pathogen interaction (Hall et al. 2010).

Furthermore, grey seals are mammals, sharing many characteristics and potential pathogens with terrestrial animals and humans. Releasing marine mammals from rescue centres may lead to the introduction of diseases acquired whilst in rehabilitation to their wider wild population. These include terrestrial diseases such as canine distemper, leptospirosis, influenza and the spread of antibiotic resistant bacterial strains (Quakenbush et al. 2009). Conversely, wild animals themselves represent potential reservoirs of infectious disease for livestock, domestic animals and humans (Rhyan & Spraker 2010; Simpson 2008). Examples of animals known to act as reservoirs of disease in Europe include red foxes (*Vulpes vulpes*) for rabies virus (Freuling et al. 2012); European badgers (*Meles meles*) for *Mycobacterium bovis* (Palmer 2013); mountain hares (*Lepus timidus*) for Louping ill virus (Hudson et al. 1997) and potentially common pheasants (*Phasianus colchicus*) and western grey squirrels (*Sciurus carolinensis*) for *Borrelia burgdorferi*, the agent responsible for Lyme's disease (Craine et al. 1997; Leonhard et al. 2010).

Finally, grey seals are exposed to a shifting environment with substantial spatial overlap with human activity and loss of natural habitat (Jussi et al. 2008). Given these characteristics, grey seals have a significant potential role as sentinels of the often cumulative, imbalances of coastal marine ecosystem health whether that consists of toxic or microbial environmental contamination, nutritional stress or prey availability, hormonal imbalances or other factors such as anthropogenic disturbance or noise (Bossart 2011; Conrad et al. 2005; Reddy et al. 2001; Stewart et al. 2008).

Assessing and understanding the disease burden of grey seals may help to define the impact that some human activities, such as farming or development of renewable energy sources, may have on this species and, conversely, the role marine mammals may play in sustaining terrestrial pathogens or introducing novel ones. In turn, this will lead to a better understanding of the Scottish coastal environment and help support effective conservation and management decisions (Hall et al. 2010). For this, it is necessary to assess the true occurrence and temporal and spatial distribution of disease within different grey seal populations and relate this to trends in the ecosystem they inhabit. Therefore this project will focus on investigating the role of infectious and parasitic disease in early morbidity and mortality of grey seal pups.

1.3 Overview of disease in Scottish grey seal populations

1.3.1 Historical overview of disease in European grey seals

Studies of the causes of non-traumatic morbidity and mortality in seal populations initially focused on bacteria and metazoan parasites, with investigation into viral, toxic and protozoal aetiologies increasing in later years as more advanced diagnostic tools became available. As with infectious diseases in other species, those of seals can be broadly classified into bacterial, viral and parasitic, and although not infectious, diseases which result as a consequence of environmental toxins, both naturally occurring and anthropogenic are also considered here.

Prior to 1980 there were very few published scientific studies focusing on disease in grey seals and these largely consisted of individual case reports (Anderson et al. 1979; Mawdesley-Thomas & Bonner 1971). The earliest systematic studies were performed in the UK between the early 1980's to late 1990's focusing on disease in grey seals in England, Wales, the Isle of Man and Scotland (Baker 1980; Baker et al. 1980) and pre-weaned pups, weaned pups and adults were examined separately. These studies resulted in the recognition of several primary causes of

mortality, such as starvation and navel-ill, or secondary (non-fatal) causes of mortality, such as conjunctivitis and mouth ulcers in pre-weaned pups (Baker et al. 1980); (Baker 1988; Baker et al. 1998). Oral ulceration, alopecia, eosinophilic peri-arteritis, panophthalmitis, mastitis, drowning, starvation and urinary calculi were among the numerous findings in older animals. Bacteriology was widely used, despite the constraints of being in the field for protracted periods (weeks), and the normal flora of female grey seals' mouths, noses, skin and nipple fossae were established on North Rona in 1985 and 1986 (Baker 1988). This flora correlated with isolates from infected lesions found in seal pups from the same colonies, largely peritonitis, navel infection and skin wounds, from which *Corynebacterium* spp., *Streptococcus* spp. and *Moraxella* spp. were most commonly isolated (Baker 1988). Comparison with previous studies on the same breeding colony site showed a shift in pathogenic bacterial flora from a predominance of Streptococcal infections to a much wider variety of opportunistic organisms between 1980 and 1985/1986, suggesting bacterial flux over time (Baker 1984; Baker 1988).

In studies of older animals, pathology was primarily associated with the respiratory and gastrointestinal tracts, consisting predominantly of pulmonary parasitism and gastric ulceration, the frequency of which increased with age (Baker 1987; Baker 1989). Nodular calcification of the walls of the pulmonary and bronchiolar arteries was a frequent finding, in all likelihood secondary to parasite migration, although no correlation was found between the lesions and the presence of pulmonary nematodes (Baker 1987).

In 1988, a mass mortality event, clinically similar to canine distemper, killed 58% of the European harbour seal population and led to the identification of a novel Morbillivirus, Phocine Distemper Virus (PDV) (Grenfell et al. 1992; Osterhaus 1989). The 1988 PDV outbreak amongst North Sea harbour seals became one of the best studied wildlife disease outbreaks and stimulated global awareness of marine mammal disease (Harkonen et al. 2006). Grey seals appeared largely unaffected by PDV a finding which, in turn, prompted investigation of the

immune response of both grey and harbour seals (Carter et al. 1992; Duignan et al. 1997). In 2002, a second outbreak of this virus occurred killing thousands of harbour seals (Harding et al. 2002).

Studies focusing on rehabilitating grey seal pups found the most common diseases to be malnutrition, respiratory and gastro-intestinal disease along with lower incidences of ocular disease, dermatitis, oral ulceration, seal pox, bacterial umbilical infection, bacterial nail bed infections and abscesses in animals found stranded on the coasts of Cornwall, Devon, the Scilly Isles and Dorset (Barnett et al. 2000).

1.3.2 Bacteria

- **Recognised Bacterial Causes of Disease**

Early investigations of seal mortalities highlighted the importance of a so-called “navel-ill complex” in pre-weaned grey seal pups which was associated with several bacterial species (*Corynebacterium* spp., *Streptococcus* spp., *Moraxella* spp., *Pasteurella* spp. and *Proteus* spp.) and presented pathologically as suppurative peritonitis and omphalitis (Baker et al. 1980). It was hypothesised that the ground substrate of the pupping areas had a significant impact on the prevalence of this condition (Baker et al. 1980), presumably by supporting differing numbers and species of bacteria and this condition is still recognised in seal colonies and rehabilitation centres around the UK (Barnett et al. 2000).

Investigation of *Salmonella* burdens in grey and harbour seals found both *S. Typhimurium* phage type 49 and *S. Bovismorbificans* to be present in both species yet with a substantially higher prevalence in grey seals (Baker et al. 1995). This was hypothesised to be due to grey seals having dirtier and denser pupping grounds as the dams give birth above the high-tide line due to the inability of their pups to swim immediately after birth (Baker et al. 1995). In contrast,

harbour seals give birth near to, or in, the water's edge and the pups are immediately able to swim (Baker et al. 1995). *Salmonella* has been associated with significant pathology such as haemorrhagic gastro-enteritis, septicaemia and acute focal hepatitis in grey seal pups (Baker et al. 1980). *Salmonella* Typhimurium phage type 49 is most commonly found in humans, which raises the possibility that the seals have become infected due to contamination from sewage effluent. However, *S. Bovismorbificans* is more host-specific with cattle being the primary reservoir, although it has also been isolated from a European otter (*Lutra lutra*) and a guillemot (*Uria aalge*) (Baker et al. 1995). *Salmonella* Typhimurium DT104, which is a virulent and drug-resistant pathogen of cattle, was isolated from a juvenile grey seal in the Moray Firth in 1998, again raising speculation of potential disease transmission between livestock and marine mammals along the coastline (Foster et al. 1998). More structured prospective studies are required to investigate this further.

- **Potentially emerging bacterial disease**

Infection with *Brucella* sp. bacteria was first reported in Scottish marine mammals (Ross et al. 1994). Subsequently, these *Brucella* strains were found to differ from species then known within this genus (Foster et al. 1996) and this led to the identification of two novel members: *B. ceti* and *B. pinnipedialis* (Foster et al. 2007). Brucellosis is both a zoonotic disease and a potential abortifacient. In cetaceans it has been associated with discospondylitis, meningo-encephalitis, blubber abscessation, splenic abscessation and occasionally shows a tropism for the genital tract (Dagleish et al. 2008; Foster et al. 2002) but its pathogenesis is, as yet, poorly understood in pinnipeds. There is little evidence that *Brucella* is a cause of disease in grey or harbour seals but *Brucella* has frequently been isolated from the lungs and spleens of these species and the potential for reproductive disruption has not yet been fully investigated due to the lack of appropriate diagnostic material (Foster et al. 2002; Nymo et al. 2011). A serological survey of marine mammals stranded around the coast of England and Wales, using competitive

and indirect enzyme-linked immunosorbent assays (ELISAs) specific for anti-*Brucella* spp. antibodies, showed positive titers in 10% of grey seals, 8% of harbour seals, 31% of harbour porpoises and 31% of common dolphins (Jepson et al. 1997). Garner *et al.* (1997) suggested a possible route of transmission by demonstrating the presence of *Brucella* spp. in the genital and digestive tracts of *Parafilaroides* spp. lungworms found in a Pacific harbour seal using immunohistochemistry and electron microscopy (Garner et al. 1997). It was also hypothesised that the Opal-eye (*Girella nigrans*) or other tidal pool dwelling fish may be a part of the transmission cycle given their role as an intermediate host for *Parafilaroides* spp. nematodes. The recent detection of *Brucella* spp. within the lungworms of a harbour porpoise (*Phocoena phocoena*) adds further support to this theory (Dawson et al. 2008). However, the transmission, pathogenesis and epidemiology of marine *Brucella* remain to be fully elucidated.

Leptospira is a major pathogen of California sea lions (*Zalophus californianus*) causing repeated fatal epizootics and reproductive failure (Gulland et al. 1996) but has also been associated with renal disease and septicaemia in harbour seals (Kik et al. 2006; Stamper et al. 1998). A suspected case of acute leptospirosis occurred in a juvenile grey seal found on the coast of Ireland which presented with jaundice (J. Barnett, personal communication). Seropositivity for *Leptospira* spp. antigens has been demonstrated in grey and harbour seals around the UK coastline with, on average, 39% of grey seals (n=180) compared to 9% of harbour seals (n=169) seropositive against one or another serovar and a much higher prevalence in grey seals from the West coast of Scotland and North Rona (up to 95%). This may suggest the presence of a host-adapted serovar in grey seals (Hall et al. 2007) but the prevalence and significance of Leptospirosis in grey seals remains poorly understood.

Several other bacterial pathogens have been recorded recently in Phocidae, namely *Escherichia coli* serotype O101, isolated from a harbour seal with fatal haemorrhagic enteritis in Northern Ireland (Watts 1995), a single case of placentitis due to *Coxiella burnetti* described in an adult

female harbour seal in California (Lapointe et al. 1999) and *Bartonella* spp. DNA was detected in pooled samples of harbour seal spleens and associated parasitic seal lice (*Echinophthirius horridus*) (Morick et al. 2009). The significance of the detection of these pathogens remains equivocal.

1.3.3 Parasites

Metazoan parasites

Metazoan parasites of grey seals are relatively well documented and include external arthropods such as the seal louse, *Echinophthirius horridus*, and the nasal mite, *Halarachne halichoeri* (Baker 1980) along with, often substantial, lungworm and gastrointestinal nematode burdens which may mask underlying pathology by their conspicuousness. Cardio-pulmonary nematode parasites, such as *Otostrongylus circumlitus* and *Parafilaroides* spp., are frequently found in the primary and secondary bronchi, leading to sub-total or even total occlusion of the bronchi (Sweeney 1974), often associated with secondary bacterial infections (Munro et al. 1992) and are a common debilitating factor in young harbour seals (Lehnert et al. 2007). The true pathological significance of these parasites is as yet unresolved. Munro *et al.* (1992) detected no associated inflammatory reaction in young harbour seals (Munro et al. 1992) but Baker *et al.* (1980 and 1998) described mortality in juvenile grey seals associated with excess mucus production in response to a heavy pulmonary parasite burden (Baker 1980; Baker et al. 1998). Furthermore, substantial vascular pathology has been reported in grey seals with lesions such as eosinophilic periarteritis or ruptured aortic aneurysms, both of which are likely to be associated with parasite migration (Baker et al. 1998).

Gastric ulceration is frequently found in grey seals, often associated with anisakid nematodes (*Pseudoterranova decipiens (sensu lato)*, *Contracaecum osculatum (sensu lato)*) and *Anisakis simplex*) causing eosinophilic, granulomatous and ulcerative gastritis which occasionally leads

to gastric perforation (McClelland 1980). Small intestinal acanthocephalan infection, due to *Corynosoma* spp. has been frequently recorded in grey seals (Baker 1987; Barnett et al. 2000) as well as occasional reports of the intestinal trematode *Cryptocotyle lingua* (Duncan 1956) and the nematode *Trichinella nativa* (Isomursu & Kunnasranta 2011) although the significance of these parasites remains in doubt.

Protozoan parasites

Toxoplasmosis is a widespread zoonotic infection caused by the protozoan parasite *Toxoplasma gondii*. While it can cause systemic disease in some mammalian species it is more commonly associated with abortion or congenital disease in its intermediate hosts, in particular sheep (*Ovis aries*) and goats (*Capra hircus*) (Innes et al. 2009). Investigation of the exposure of Scottish seals to *T. gondii* found that 23.4% of grey seals and 5.4% of harbour seals were seropositive (Cabezón et al. 2011). A similar study in Canadian pinnipeds showed that 9% of grey seals, 9% of harbour seals and 2% of hooded seals (*Cystophora cristata*) were seropositive (Measures et al. 2004). Furthermore, positive antibody titres to both *T. gondii* and *Neospora caninum*, a closely related parasite which is a major cause of abortion in domestic cattle (Dubey et al. 2007), have been detected in harbour seals from the coast of Japan (Fujii et al. 2007).

Experimental infection of captive grey seals with *T. gondii* has been shown to result in seroconversion, detected by a modified agglutination test (Gajadhar et al. 2004). Cats fed infected seal tissues passed *T. gondii* oocysts in their faeces suggesting that grey seals are capable of acting as an intermediate host and/or marine reservoir for this pathogen. Investigating the ecology of this pathogen in southern sea otters (*Enhydra lutris nereis*), Conrad et al. (2005) discuss potential sources of exposure such as ingestion of filter-feeding marine invertebrates containing tissue cysts, vertical and direct transmission routes (Conrad et al. 2005). Recently *Toxoplasma* oocysts have been shown to survive for 24 months in salt water at 4°C (Lindsay et

al. 2003; Lindsay & Dubey 2009) supporting the hypothesis that the infection could result from direct contamination from land to sea.

A novel *Sarcocystis* species, tentatively named *S. pinnipedi*, has been associated with fulminant necrotising hepatitis in grey seals in the North Atlantic and Pacific oceans (Haman et al. 2013; Haman *et al.*, unpublished). Non-suppurative meningoencephalitis and non-suppurative myocarditis have been described in harbour seals with *Sarcocystis neurona* (Lapointe et al. 1998; Miller et al. 2001b). Dual infections of *S. neurona* and *T. gondii* have been described in other species of stranded marine mammals in the Pacific Northwest (Gibson et al. 2011; Miller et al. 2001b). Coinfection was demonstrated to be more frequently associated with mortality and protozoal encephalitis than single infections, indicating a role for polyparasitism in disease severity (Gibson et al. 2011).

Giardia, a zoonotic, waterborne intestinal parasite of major human health concern (Thompson 2004), has been isolated from grey seals in eastern Canada (Measures & Olson 1999). Thus, grey seals represent potential reservoir hosts for these pathogens but the pathogenicity of this parasite to the seal is unknown.

1.3.4 Viruses

Several viral species have been identified as causes of significant morbidity and mortality among pinnipeds (Martina et al. 2003; Osterhaus et al. 1985; Osterhaus et al. 1994; Osterhaus et al. 2000; Osterhaus & Vedder 1988).

Morbillivirus

As mentioned previously, two successive epizootics of Phocine Distemper Virus (PDV) spread throughout seals resident along Northern European coastlines in 1988 and 2002 (Barrett et al. 1992; Hall 1995; Osterhaus & Vedder 1988; Pomeroy et al. 2005) leading to estimated mortality

rates of up to 52% of harbour seals in areas such as the Wash in England in 1988 and lower, but nonetheless significant, mortality rates in 2002 (<22%). The aetiology of the initial outbreak was unknown. However, the affected harbour seals developed a striking fibrino-necrotising broncho-interstitial pneumonia with syncytia formation, type II pneumocyte hyperplasia and eosinophilic intracytoplasmic and intranuclear viral inclusion bodies. Additionally, lymphodepletion of the spleen, lymph nodes, thymus and Peyer's patches occurred, as well as occasional cases of non-suppurative demyelinating encephalitis. All these lesions were similar to those found in cases of canine distemper virus (CDV) infections in dogs (Baker 1992; Kennedy et al. 1989). Morbillivirus antigen was identified within lesions using immunohistochemistry leading to the discovery of PDV (Kennedy et al. 1989). Lungs frequently showed secondary bacterial infections with agents such as *Bordetella bronchiseptica*, *Corynebacterium* spp. and streptococci along with moderate to large burdens of lungworms (Baker & Ross 1992). The grey seal population was much less affected by the PDV epizootics but was shown to become infected and allow replication of the virus, leading to speculation that this species had an active role in the spread of PDV during an epidemic (Hammond et al. 2005b).

Mortality in the grey seal population was low during both epizootics and post-mortem examinations of grey seals failed to find characteristic lesions of PDV. However, the birth rate of grey seal pups in 1988 fell by approximately 12% compared to the previous years' averages and several aborted fetuses washed up on beaches of North West England and North Wales, suggesting that the virus may induce abortions in this species (Harwood et al. 1989). Abortion due to PDV infection has been recorded in harbour seals (Osterhaus 1989).

Using a CDV virus-neutralisation test, a rapid and strong humoral immune response to PDV was detected in grey seals post-1988 with 96, 59 and 83% of the population showing positive titres in 1988, 2001 and 2002 respectively compared to 0% prior to this (Pomeroy et al. 2005). Harbour seals showed statistically significantly lower virus neutralising titres to CDV antigen

despite the higher mortality rate in this species, possibly explaining their higher disease susceptibility. Harbour seals with the respiratory form of Morbillivirus infection showed very weak to no humoral immune response to PDV infection, whereas those with chronic encephalitis, similar to that seen in dogs with CDV infection, have some humoral immune response (Duignan et al. 1997). The origin of these two outbreaks was not fully elucidated but an arctic seal species, such as the harp seal (*Pagophilus groenlandicus*) or hooded seal which can carry PDV asymptotically, may have introduced it into European seal populations (Hall et al. 1992; Hall 1995). Although not clinically affecting grey seals, the Morbillivirus outbreaks in harbour seals in 1988 and 2002 highlighted a lack of baseline data on grey seal pathogens.

Influenza A and B

The first records of influenza epizootics in any pinniped species were cases of haemorrhagic and interstitial pneumonias in harbour seals in New England associated with influenza A virus (Geraci et al. 1982; Hinshaw et al. 1984). Although the pathology in each epizootic was similar, the strain of influenza A differed: H7N7 in the 1979-80 outbreak and H4N5 in the 1982-83 outbreak (Hinshaw et al. 1984). Later reports described Influenza A strains H3N3 and H4N6 isolated from harbour seals with interstitial pneumonia (Callan et al. 1995). These findings suggest that seals are susceptible to Influenza A viruses, and these seem to originate from avian reservoirs (H4N5, H7N7, H4N6 and H3N3) (Callan et al. 1995). Given that seals are mammals and that viruses capable of replicating in seals may be more adapted to mammalian than avian hosts, the potential for genetic reassortment with transmission to humans must be considered (Callan et al. 1995).

Influenza B virus, a human pathogen, was first isolated from 12 juvenile harbour seals in a Dutch rehabilitation centre in 1999 which all presented with clinical signs of respiratory disease (Osterhaus et al. 2000). The virus induced cytopathic changes when in cultured canine kidney cells and was identified as an orthomyxovirus consistent with Influenza B after electron

microscopy, RT-PCR and sequence analysis (Osterhaus et al. 2000). A retrospective analysis of sera from seals previously held in the rehabilitation centre showed no positive titres to Influenza B virus prior to 1995 and 2% seropositivity from 1995 including both harbour seals and grey seals. The authors did not comment on the pathogenicity of the virus. However, given the similarities with a human strain, they hypothesised that it may have been introduced by humans around 1995 and possibly represented a reservoir of human disease (Fouchier et al. 2001; Osterhaus et al. 2000). A second wave of seropositivity was detected in 2010, suggesting that Influenza B virus infection of seals is a recurrent process rather than an isolated incident, with seal serum samples suggesting exposure to a different strain of influenza B virus than that found in 1999 (Bodewes et al. 2013a).

Poxvirus

Proliferative lesions of the skin and oral mucosa of grey seals have been attributed to a novel group of marine poxviruses, bearing much similarity to terrestrial parapoxviruses such as those seen in contagious pustular dermatitis (orf) of sheep and goats and bovine papular stomatitis. This phocine poxvirus was grown in grey seal kidney cells (Nettleton et al. 1995) and has been found to be zoonotic, leading to papular skin lesions similar to “milker’s nodule” (Clark et al. 2005; Hicks & Worthy 1987). In grey seals, the lesions arise in young animals and are frequently exacerbated by stress as they often appear a few weeks after entering a rehabilitation centre (Hicks & Worthy 1987). Infection manifests as plaque-like to nodular areas of hyperkeratosis and parakeratosis with ballooning degeneration of keratinocytes with eosinophilic intracytoplasmic, and occasionally intranuclear, viral inclusion bodies (Muller et al. 2003). The clinical significance is not fully known but this condition is thought to cause a highly infectious, self-limiting disease which can leave areas of scar tissue or alopecia following resolution. Two grey seals were described with simultaneous infections of poxvirus and calicivirus as detected by electron microscopy (Stack et al. 1993). These seals presented clinically with peri-ocular epidermal ulceration and one seal had shallow ulcers along the

mucocutaneous margins of the lips and lateral and ventral aspect of the tongue, similar to that seen with Poxvirus alone. The significance of the presence of calicivirus particles in these cases is as yet unknown.

Herpesviruses

Two distinct Herpes viruses have been described in seals. Phocid herpesvirus-1 (PhHV-1), has been found in both grey and harbour seals in the Eastern Atlantic and Pacific Oceans and identified as an alphaherpesvirus (Borst et al. 1986; Gulland et al. 1997; Himworth et al. 2010; Martina et al. 2002). PhHV-1 causes moderate to severe clinical respiratory disease in younger animals, with more mild disease and correspondingly less severe clinical signs to sub-clinical infections in older animals (Martina et al. 2002). Grey seals have been reported to develop less severe clinical signs than harbour seals (Martina et al. 2002). In harbour seals the pathology and pathogenesis of this virus differs between geographically distinct populations. Eastern Atlantic harbour seals develop hepatic necrosis, interstitial pneumonia, degenerative changes in the renal tubular epithelium, oral mucosal ulceration and lymphodepletion (Borst et al. 1986), whereas Pacific harbour seals develop adrenocortical necrosis and multifocal hepatic necrosis with intranuclear viral inclusion bodies (Gulland et al. 1997).

There is evidence of horizontal and, most likely, vertical transmission of PhHV-1 in both free-ranging Pacific harbour seals in California and those in rehabilitation centres, with the possibility of viral latency and reactivation in pregnant females (Goldstein et al. 2004).

Morbidity and mortality due to PhHV-1 vary from high (Gulland et al. 1997; Osterhaus et al. 1985) to low (Goldstein et al. 2004) between reports, suggesting the possibility that extrinsic factors, such as stress, may affect the severity of the clinical manifestation of the disease in harbour seals (Goldstein et al. 2004). A study on tissue distribution of PhHV-1 in naturally infected harbour seals detected viral DNA most frequently from the ileo-caeco-colic junction, bronchial lymph node, spinal ganglia and trigeminal ganglia using PCR techniques (Goldstein et

al. 2005). Mononuclear leucocyte-associated viraemia followed by spread of the virus to regional lymph nodes and mucosa associated lymphoid tissue (MALT) prior to dissemination to parenchymal organs has been suggested as a possible course of progression of infection (Goldstein et al. 2005). Methods for the detection and localisation of viral antigen using immunohistochemistry or of viral DNA using in-situ-hybridisation (ISH) remain to be developed.

A PhHV-1 specific ELISA was developed to measure harbour seal humoral immune response over time and results were correlated with neonatal mortality (King et al. 2001). In one, study prevalence of exposure to PhHV-1 in Pacific harbour seals was estimated at 37.5% of pre-weaned pups, 87.6% of weaned pups and 99% of subadults and adults, based on positive titres, suggesting that PhHV-1 is endemic in North American harbour seal populations (Goldstein et al. 2003). Although a substantial body of work exists focusing on the harbour seal, very little is known about the pathogenicity and pathogenesis of this virus in the grey seal.

Phocid herpesvirus-2 (PhHV-2) has been isolated from European grey seals, European harbour seals and Pacific harbour seals (Harder et al. 1996; Lebich et al. 1994) and classified as a putative gammaherpesvirus (Harder et al. 1996). Genetic characterisation of different PhHV-2 isolates suggests that grey seals and harbour seals are infected with distinct gammaherpesviruses but they may circulate between the two species (Martina et al. 2007). The pathogenicity of the virus is, as yet, unknown. Seals infected with PhHV-2 have occasionally shown clinical signs of respiratory disease (Lebich et al. 1994; Martina et al. 2003). However, the significance of this observation is doubtful given the high prevalence of lungworm in these animals possibly confounding the clinical picture. PhHV-2 grows in several different cell lines and displays cytolitic effects in seal peripheral blood monocytes grown *in vitro*, suggesting the possibility of an immunosuppressive effect (Martina et al. 2003). Experimental infection of inbred BALB/c mice with PhHV-2 led to increased incidence of myoepithelioma development

following cyclosporine-A treatment suggesting an oncogenic potential for this virus (Martina et al. 2007).

Sporadically reported viruses

Several other viruses have been identified in true seals such as a coronavirus associated with acute necrotising enteritis and pulmonary oedema in three harbour seals (Bossart & Schwartz 1990), Eastern equine encephalitis virus in a captive harbour seal presenting with seizures, meningo-encephalitis and adrenal cortical necrosis (McBride et al. 2008) and rabies virus in a ringed seal (*Pusa hispida*) and in Norway presenting with an “oedematous skin condition”, “yellowish-green exudate from the eyes and mouth” and aggression (OEdegaard & Krogsrud 1981). The biological significance of these cases to the respective populations is debatable.

1.3.5 Toxins

Reports of harmful algal toxin incidents in marine mammals have increased in recent years. The agent responsible for amnesic shellfish poisoning, domoic acid, was first reported to be present in sea lions in 1998 and is associated with acute neurological clinical signs such as persistent seizures (Gulland et al. 2002). The pathology is one of ischaemic neuronal necrosis, laminar microvesicular degeneration of the hippocampal neuropil, myocardial necrosis and haemorrhage in acute cases and bilateral hippocampal atrophy in animals dying several months after the toxic insult. More recently, the role of domoic acid on premature parturition in California sea lions has been studied and foetal brain oedema was a frequent finding suggesting a possible causal effect (Goldstein et al. 2009). Low levels of domoic acid have been reported in the urine and faeces of live-captured harbour seals off the Scottish coast but association with disease remains to be proven (Hall & Frame 2010).

1.3.6 Neoplasia

The most significant neoplasm found in pinnipeds is the urogenital carcinoma of California sea lions which has been associated with a novel gammaherpesvirus (Newman & Smith 2006).

Therefore the potential for neoplasms to arise secondary to infectious diseases cannot be excluded. Within the known pathogens of grey seals, gammaherpesviruses and poxviruses would be putative agents worthy of further investigation.

In grey seals, neoplasms have only been described in the uterus. There are several reports of uterine leiomyoma, commonly found in Baltic grey seals, thought to be linked to PCB pollution (Bäcklin et al. 2003; Baker 1989; Mawdesley-Thomas & Bonner 1971) and a single report of uterine squamous cell carcinoma and uterine carcinoma (Mawdesley-Thomas & Bonner 1971).

Neoplasia is relatively rare in other true seals. Reports include a dermal melanoma in a harbour seal (Morick et al. 2010), a primary meningeal T cell lymphoma in a harbour seal (Labrut et al. 2007), biliary adenocarcinoma in Northern Elephant seals (*Mirounga angustirostris*) and adrenal tumours, an ovarian granulosa cell tumour and leiomyoma of the uterine cervix in Southern Elephant seals (*Mirounga leonine*) (Newman & Smith 2006).

1.3.7 Surveillance programs

After the first Morbillivirus epizootic in seals, the Scottish Marine Mammal Stranding Scheme was rapidly established in 1992 at the SAC Veterinary Services Wildlife Unit in Inverness. This project manages the Scottish operation of the UK wide Cetacean Strandings Investigation Programme (CSIP) and assesses the numbers and trends of stranded marine mammals by investigating the potential causes of death, identifying and determining the prevalence of disease and contaminants in marine mammals and improves knowledge of life history

parameters in order to identify any substantial new threats to their conservation status (The Scottish Agricultural College 2000).

1.3.8 Techniques available to investigate infectious disease and their limitations

Investigation of infectious disease is a complex process and relies on several complementary approaches which may include: 1) detecting pathology or host response to a pathogen; 2) detecting the presence of a specific pathogen; 3) showing evidence of previous exposure to a pathogen; 4) studying of the pattern of disease within a population. The limitations of each approach should be constantly kept in mind, and ideally, a combination of techniques used.

The host response to a pathogen can be monitored by methods such as clinical examination, necropsy or histopathology but, although fundamental in disease investigation, are often not sufficiently specific to allow an unequivocal diagnosis.

Detection of a specific pathogen can be performed directly using culture methods, with specific media for agents such as bacteria or fungi or using cell-culture for viruses. Identification of proteins (Western blot) or nucleic acids (polymerase chain reaction (PCR), real-time PCR (qPCR), Southern blot) are also methods of direct identification of agents. However, identification of a pathogen, while a crucial element of disease investigation, does not necessarily imply causality.

Previous exposure to an agent can be measured using serology, detecting antibodies produced by the host in response to a challenge. While indicating exposure, a positive antibody titre to a specific pathogen does not imply current infection, nor that any clinically apparent disease has occurred. In addition, antibody titers in neonates of many species can be affected by the presence of maternally transferred antibodies.

The pattern of disease within a population relies on epidemiological assessment of host susceptibility traits (genetic, nutritional etc.) or environmental parameters. Finally, techniques such as immunohistochemistry (IHC) or in-situ hybridisation allow simultaneous assessment of pathogen presence and host response and are therefore valuable, if rare, tools for disease investigation.

1.4 Aims of the study

The aims of this study were to compare and contrast causes of morbidity and mortality in free-living grey seal pups from a breeding colony in the North Sea with those seen in the same, then recently established, colony over 25 years previously.

The first objective was to describe the causes of neonatal mortality in a well-established grey seal breeding colony, proving a baseline of what represents “normal” neonatal mortality in this population. Findings were compared to causes of mortality described in this same population over 25 years previously and to causes of mortality observed in stranded grey seal pups admitted to a rehabilitation centre (Chapter 3). To do this, extensive systematic post-mortem examinations were performed, in parallel with extensive bacteriology and screening for known viral and protozoal pathogens of grey seals (Chapters 3 and 6). Correlation of gross and histopathological lesions with the presence of particular pathogens aimed to further the understanding of the pathogenesis and significance of individual and multiple infectious agents in the grey seal population (Chapters 3 and 6).

The second objective was to investigate the potential use of grey seals as sentinels of coastal marine health by screening for several pathogens of putative anthropogenic origin. The presence of the protozoan parasites *Toxoplasma gondii* and *Neospora caninum* was investigated in Chapter 6. The presence of the faecal bacteria *Salmonella* spp. (Chapter 4) and *Campylobacter*

spp. (Chapter 5) was investigated, along with detailed studies of their pathogenicity. To help elucidate the origin of isolates recovered from grey seals and their relationship with known terrestrial and human isolates, extensive phenotyping, molecular typing and comparative genomics of bacterial isolates were performed.

Chapter 2 Sampling of grey seal pups and yearlings – Study sites, sampling methods and samples

2.1 Sites

2.1.1 Isle of May

The Isle of May is located in the outer Firth of Forth approximately 8 km from the Scottish mainland (56.18°N; 2.55°W) (Figure 2-1) and is currently maintained as a Scottish Natural Heritage nature reserve. It is the largest grey seal breeding colony on the East coast of Scotland and the fourth-largest breeding colony in the UK, contributing approximately 4.5% of annual UK pup production (Thompson & Duck 2010). Designated as a Special Area of Conservation (SAC), the site forms part of the North Sea grey seal breeding grounds along with the colonies at Fast Castle, the Farne Islands, Donna Nook, Blakeney point and East Anglia (<http://jncc.defra.gov.uk/protectedsites/sacselection/sac.asp?EUCode=UK0030172>).

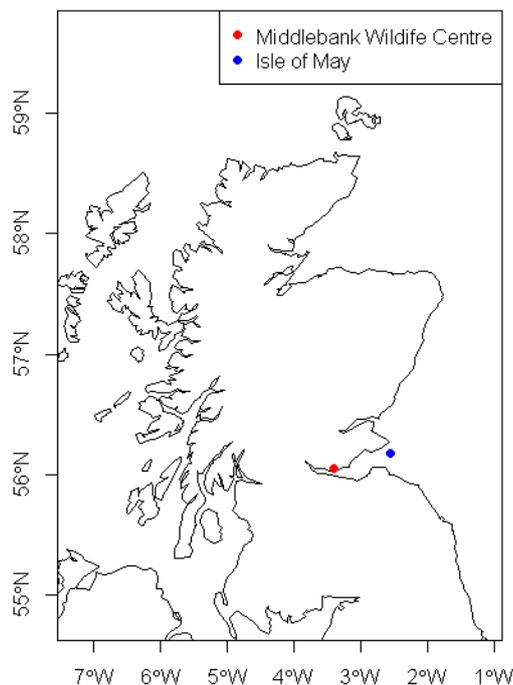


Figure 2-1 Location of the two main study sites: Grey seal breeding colony: The Isle of May; Rehabilitation centre: Scottish SPCA National Wildlife Rescue Centre, Middlebank Farm, Dunfermline, Fife

The first reported grey seal pup births on the Isle of May occurred in the 1950s (Baker 1988, Eggeling, 1985). Since then numbers have gradually increased with around 30 pups born in 1977 (Harwood & Wylie 1987) and an estimated 2356 pups born in 2012 (C. Morris, SMRU, pers. communication) (Figure 2-2 and Figure 2-4).

There has been an exponential increase in pup production in North Sea grey seal breeding colonies since regular surveys began in the 1960s (Figure 2-2 and Figure 2-3). The increase is slowing down at the Isle of May, largely to the benefit of neighbouring colonies such as Fast Castle which has seen its pup production increase from 236 in 1997 to over 1200 in 2008 (Figure 2-2). This growth of 19% *per annum* (p.a.) at Fast Castle is higher than that expected in a closed population (12% p.a.) implying recruitment from other breeding colonies such as the Isle of May (Thompson & Duck 2010).

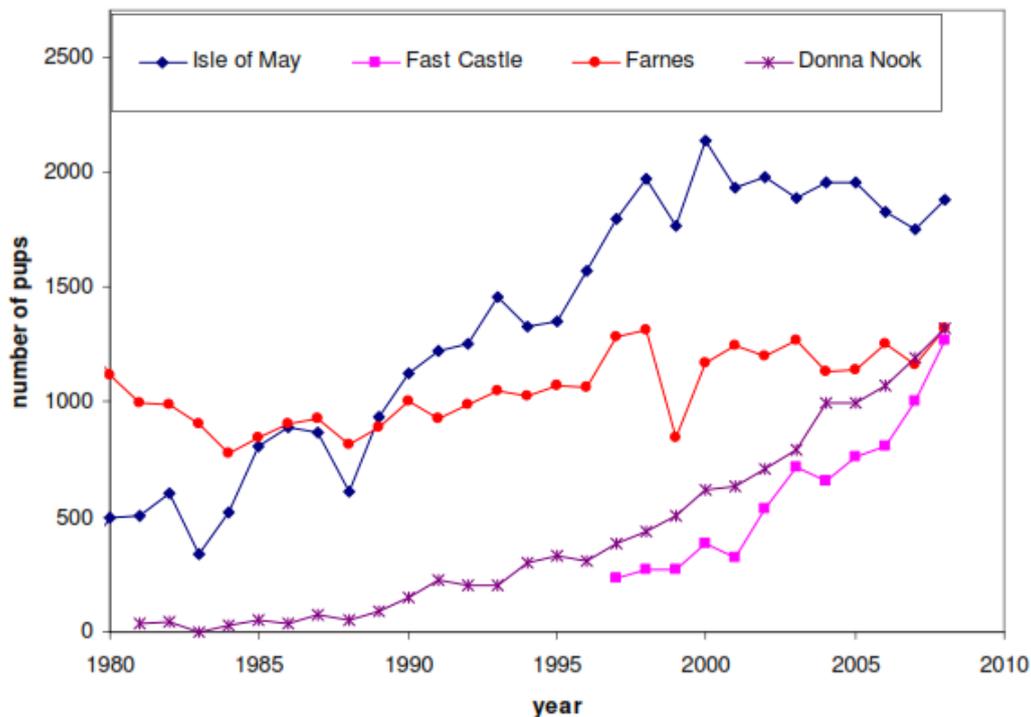


Figure 2-2 Grey seal pup production at the four main breeding colonies in the North Sea: Farne Islands, Fast Castle, Isle of May and Donna Nook. Source: Sea Mammal Research Unit (Thompson & Duck 2010)

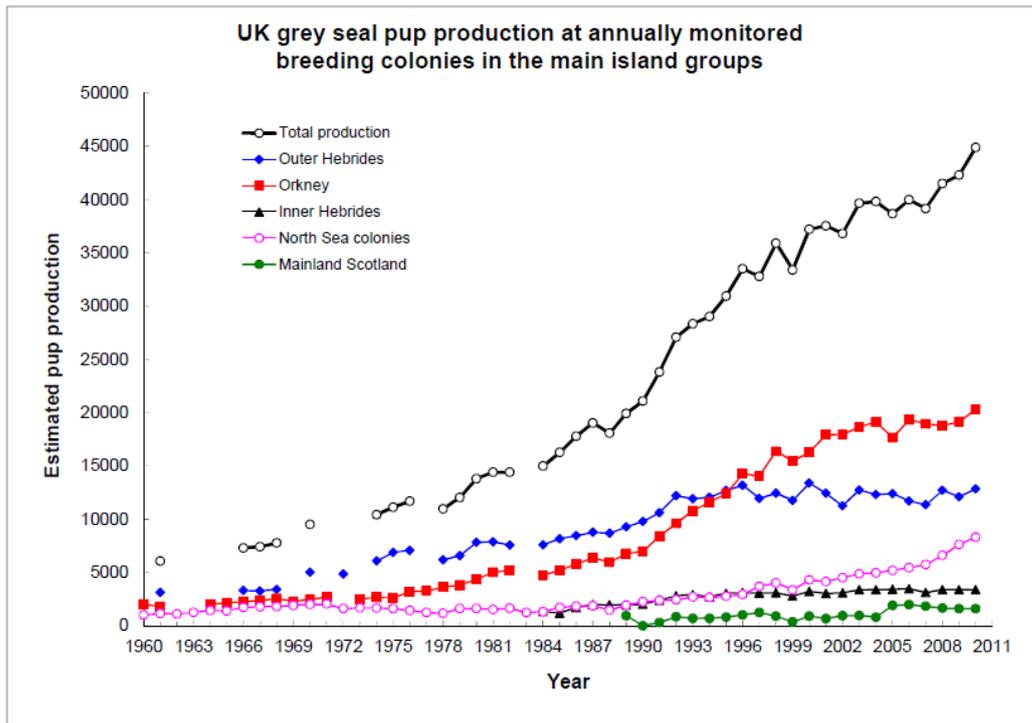


Figure 2-3 Grey seal pup production trajectories from 1960 to 2010. Source: Sea Mammal Research Unit (Duck & Morris 2011)

The Isle of May shows evidence of delayed recruitment of females to the breeding population, with re-sightings of tagged females at sites such as Donna Nook and Fast Castle (Pomeroy et al. 2010). This may be consistent with the effects of density dependence which is suspected on the Isle of May, and contribute to the stabilisation of pup production seen in recent years (Pomeroy et al. 2010).

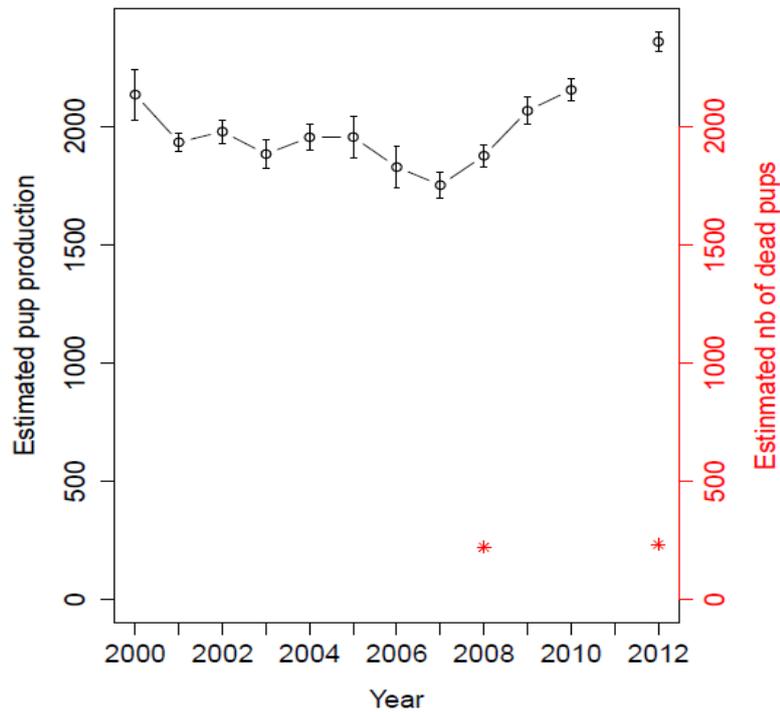


Figure 2-4 Estimated pup production on the Isle of May from 2000 to 2012 based on aerial count surveys (data: C. Morris, SMRU). N. B.: No survey was carried out in 2011. Asterisks represent land based counts of dead pups, thus a lower (conservative) estimate of pup mortality, on the Isle of May for the 2008 and 2012 pupping seasons of 12.4% and 9.9% respectively.

In parallel with the substantial expansion of the Isle of May breeding colony, the area occupied by seals has significantly increased and sites formerly unoccupied by grey seals have become colonised over the years (Pomeroy et al. 2000a). Indeed, the local animal density has declined rather than increased, with the expansion of the colony, largely through gradual peripheral expansion, thus establishing new pupping sites on a variety of different substrates (Pomeroy et al. 2000a).

Substrate variation on the Isle of May

To assess the effect of substrate on the cause of death and presence/absence of specific pathogens, the location and substrate type were recorded. Each location corresponded to a substrate type: grassy; tidal boulder beach; rocky stagnant pools or mixed. Grassy areas included Tarbet slope, Rona top and pups high up on the Loan (see Figure 2-5 for map of Isle of

May sampling locations). The Kirkhaven site is composed of multiple substrate types including shingle beach, mud, rocky pools and grass so attributing these pups to one substrate type was not possible. For these pups the substrate type was recorded as “Mixed”.

Live free-ranging pups were only sampled from each of three locations with very different substrate characteristics: Pilgrim’s haven (56.1815° N; 2.5511°W); Tarbet slope (56.19°N; 2.5621°W) and Rona rocks (56.1909°N; 2.5618°W).

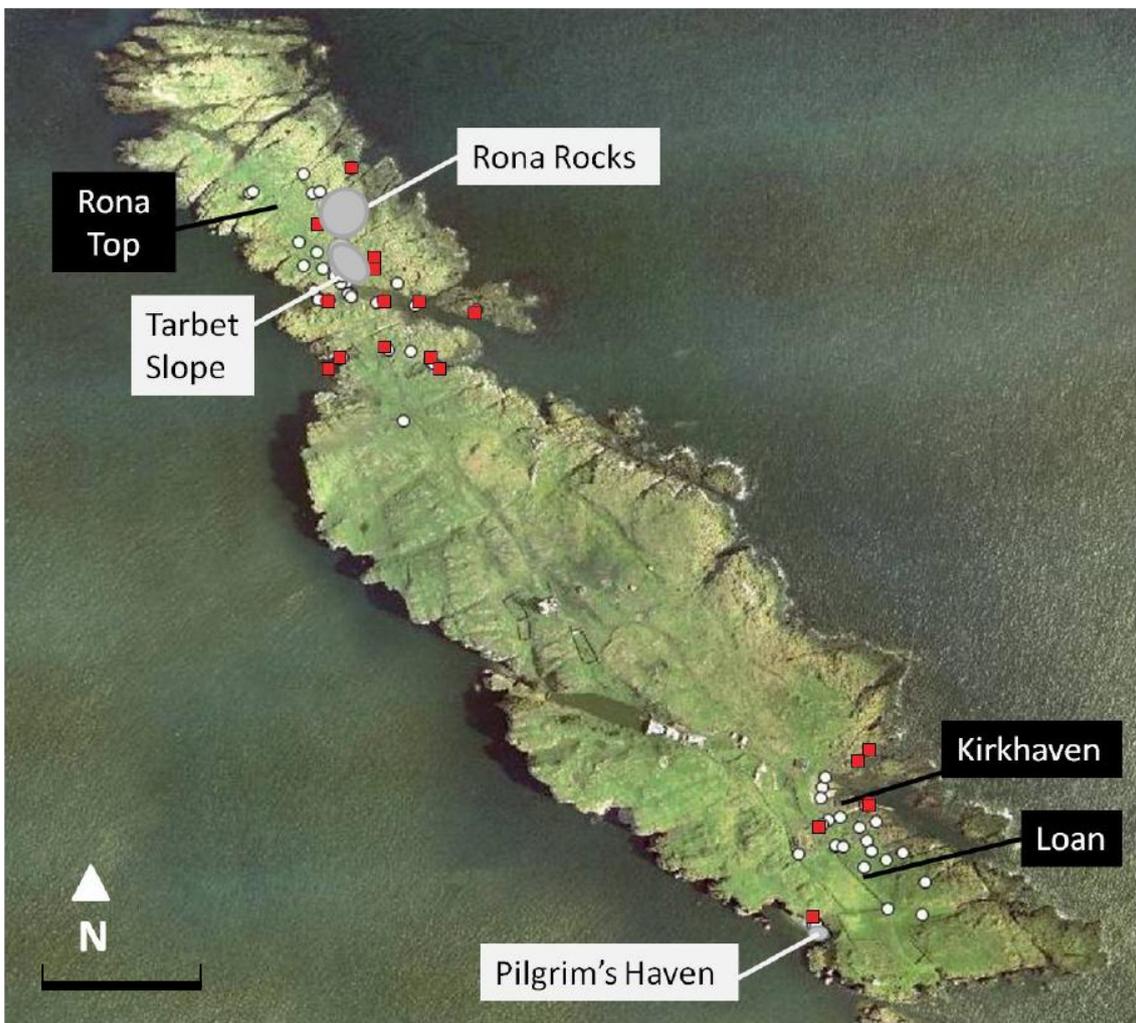


Figure 2-5 Map of Sampling locations on the Isle of May; Grey circles: Sampling sites of live grey seal pups; White circles: location of dead grey seal pups; red squares: sampling site of live yearlings. Scale bar: 200 metres.

Chapter 2 – Sites and Sampling

The tidal boulder beach of Pilgrim's haven (Figure 2-6) consists of medium sized, volcanic, olivine-dolerite (greenstone) boulders, interspersed with a small amount of coarse sand, delimited either side and at the back by steep greenstone sea cliffs (Eggeling 1960). The beach is almost entirely covered by the in-coming tide, leaving only a narrow strip of beach and small rocky caves as substrate for the pups at high tide. Throughout this twice daily process, pups are potentially exposed to sea water, but are also subjected to significant displacement and crowding.

Tarbet slope (Figure 2-7) consists of a relatively open grassy slope, interspersed with small, rocky outcrops, which becomes increasingly muddy as the pupping season progresses.

Movement of adult grey seals consists predominantly of regular commutes to small pools or to the sea at Rona cut, leading to occasional displacement and interactions of pups in this area with adult grey seals or other pups.

Rona rocks (Figure 2-8), on the other hand, is an extensive, slightly sloping moonscape of undulating greenstone (Eggeling 1960), dissected by numerous fault lines and forming small freshwater pools which become heavily contaminated with seal faecal matter, detritus and dead pups over the course of the pupping season. Adult movements are restricted to commutes, often to the small, murky freshwater pools and occasionally to the sea. Pups tend to be situated on rocky to grassy ground, often located within a depression or cleft and movements and interactions are relatively restricted.

With the exception of the far North of the island, access to which was restricted to limit disturbance to the colony, the location of sampling sites and carcass collection (Figure 2-5) mirrored the distribution of grey seals throughout the Isle of May.



Figure 2-6 Pilgrim's haven. Tidal boulder beach delimited by greenstone sea-cliffs.

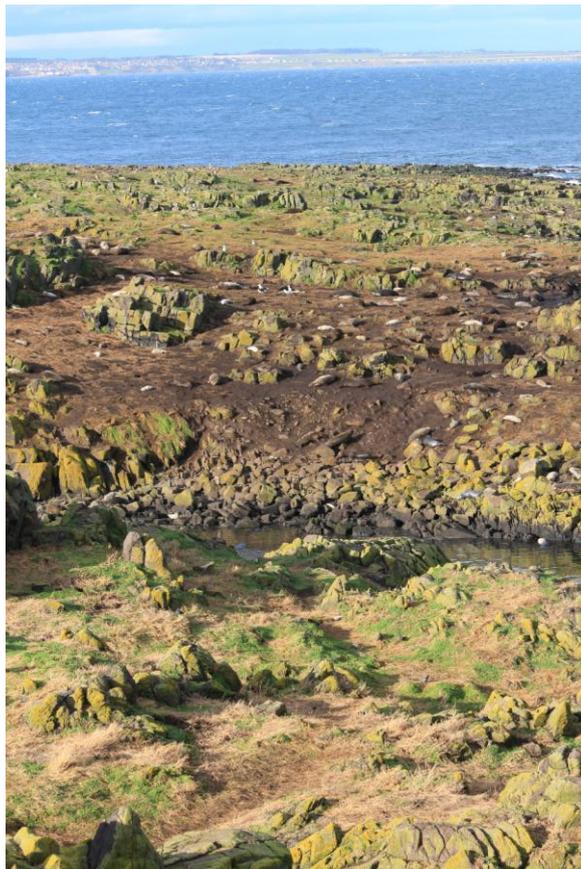


Figure 2-7 Tarbet slope. Muddy, grassy slope interspersed with small, rocky outcrops.



Figure 2-8 Rona Rocks. Sloping greenstone dissected by numerous clefts and depressions, forming freshwater pools.

Time period

To assess the changes in cause of death or pathogen carriage over the course of the 2011 breeding season, three time periods were defined, each spanning approximately 12 days: “Early pupping season” ranged from the 28th October 2011 to 9th November; “Mid pupping season” ranged from 10th November to 20th November and “Late pupping season” ranged from 21st November to 3rd December. In addition, any stranded grey seal pups admitted to the rehabilitation centre (see below) after the 4th December were assigned to the “other” category.

2.1.2 Scottish SPCA National Wildlife Rescue Centre

The Scottish SPCA National Wildlife Rescue Centre is a wildlife rehabilitation centre run by the Scottish Society for Prevention of Cruelty to Animals (SPCA) and was located at Middlebank Farm in Dunfermline in Fife, Scotland at the time of this study (Figure 2-1). Being the largest rehabilitation centre of this type in Scotland, it receives stranded and injured grey seal pups from all around the Scottish coastline throughout the breeding season (Figure 2-9). Since April 2012 the centre has been relocated to Fishcross in Clackmannanshire.

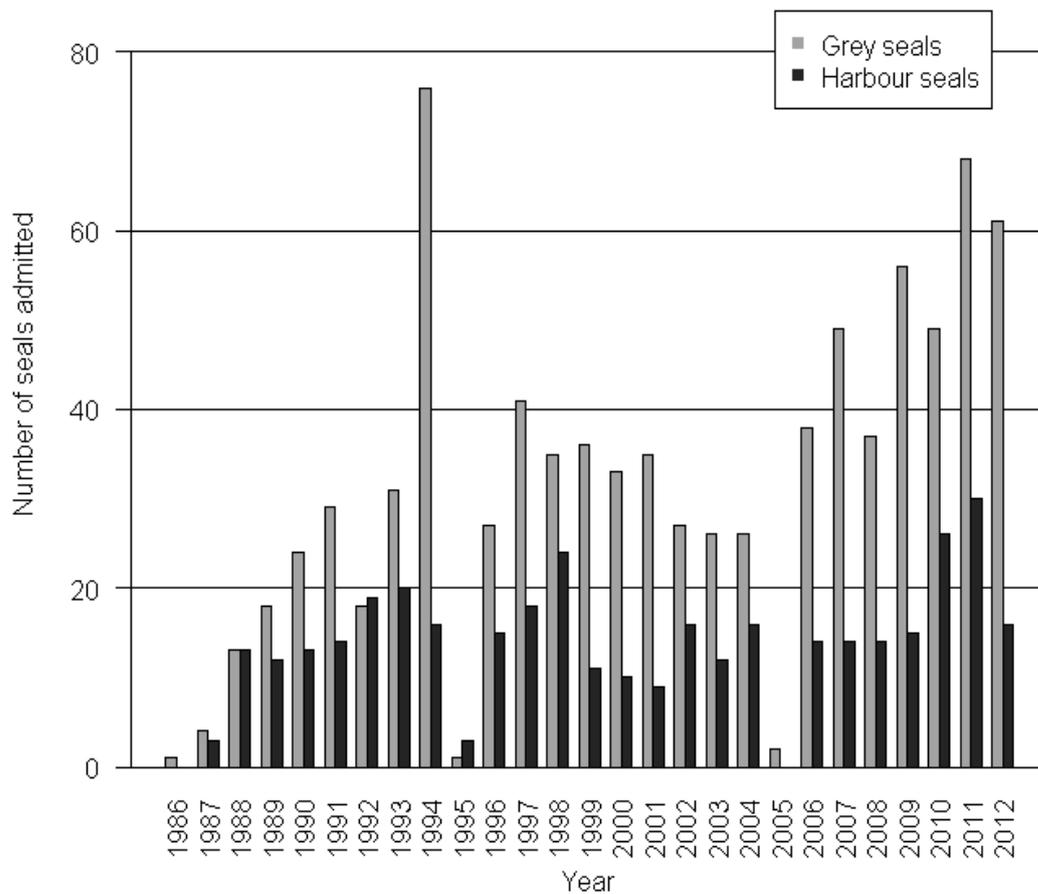


Figure 2-9 Number of grey and harbour seals admitted to the Scottish SPCA National Wildlife Rescue Centre between 1986 and 2012 per calendar year

Rehabilitation success rate for grey seals surviving the first 24h after admission is currently estimated at 66% (Pizzi et al. 2012). All stranded grey seal pups used in this study were found at various locations along the Scottish coastline (specified in Figure 2-10) during the 2011 breeding season before being transported to Scottish SPCA National Wildlife Rescue Centre for rehabilitation.

2.2 Capture methods and sampling

2.2.1 Live animals

- **Isle of May**

All sampling of live animals was carried out under UK Home Office Project (No. 60/4009) and Personal Licences as issued to the Sea Mammal Research Unit under the Animals (Scientific Procedures) Act, 1986.

Sampling of live pups and yearlings was carried out in teams of 3-4 people in order to restrict time spent within the colony and thus disturbance. Only animals in apparently good health were selected for the study. Any visibly/subjectively underweight pups were not considered. When possible, pups whose mother was absent (commuting to sea or pool) were selected in order to limit disturbance to the mother-pup pair. When present, adult females were distracted and restrained using protective boards. In addition, pups with mothers demonstrating a strong flight response were not selected for inclusion in the study so as to limit the chance of abandonment following sampling. Capture and restraint of the pups was carried out using specialised bags and no pups or yearlings were sedated or anaesthetised for the purposes of this study.

- **Scottish SPCA National Wildlife Rescue Centre**

Sampling kits were assembled and provided to the Scottish SPCA National Wildlife Rescue Centre prior to the pupping season. Each kit consisted of two rectal swabs, one nasal swab, two stamped addressed insulated envelopes, ice-packs and two information forms containing a unique identifier for each case. Stranded grey seal pups were sampled within 24h of arrival at the rehabilitation centre as part of the routine health assessment procedure.

2.2.2 Dead pups

Isle of May

All pupping sites, with the exception of the far North of the island (North of the North Horn and Silver Sands) were visited every 24 -72h, often in association with other sampling/research activities, to limit disturbance to the colony. Monitoring was carried out visually using binoculars to locate dead pups. Freshly dead carcasses were collected and transported back to the laboratory. Any carcasses showing significant bird scavenging or with obvious post-mortem degeneration were excluded from the study as microbiological assessment was deemed to be too altered and histological assessment would be compromised. Any pup known to be dead for over 48h without the opportunity of collection or due to delay in processing was also excluded from the study. A unique identifier was attributed to each animal and the exact location (latitude and longitude to the closest 0.0001°) was calculated using GIS (Geographic Information Software) software Manifold version 8.0 (<http://www.manifold.net/index.shtml>).

Scottish SPCA National Wildlife Rescue Centre

Greys seal pups which died or were euthanised on humane grounds at the Scottish SPCA National Wildlife Rescue Centre during the study period were submitted to the Moredun Research Institute for post-mortem examination. Any pup known to be dead for over 48h without the opportunity of collection or due to delay in processing was excluded from the study.

2.3 Collection of morphometric data and samples

Morphometric data

A unique identifier was attributed to each animal consisting of a two parts: One or two letters identified the group sampled (see below) and a three digit number identified the individual pup within the group in chronological order of sampling (ex: CL001; R005).

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A = “Arrival rehab”: Stranded live grey seal pup on arrival at the Scottish SPCA National Wildlife Rescue Centre

R = “Rehab dead”: Stranded dead grey seal pup (died or euthanised) at the Scottish SPCA National Wildlife Rescue Centre

CL = “Colony-live”: Free-ranging live grey seal pup on the Isle of May

CD = “Colony-dead”: Free-ranging dead grey seal pup on the Isle of May

Y = “Yearling”: Free-ranging live grey seal yearling on the Isle of May

The following data were recorded: Sex, pup developmental stage as described by Kovacs and Lavigne (Kovacs & Lavigne 1986), mass using spring balanced scales (Salter Industrial Measurements Ltd., West Bromwich, UK) in a pup-bag (to the nearest 100g); length (nose to tail) (to the nearest 5mm), girth immediately posterior to the axilla (to the nearest 5mm) and the presence of any external anomalies (bites, areas of alopecia, nasal discharge, ocular discharge, presence/absence of umbilical cord). For stranded grey seal pups presented to the Scottish SPCA National Wildlife Rescue Centre no distinction was made between pup stages I to III, all of which were recorded as “Whitecoat”. Average weights were expressed as mean \pm standard deviation.

The approximate location of stranded grey seal pups was recorded by the rehabilitation centre and converted into a latitude and longitude using iTouchMap.com free on-line software (<http://itouchmap.com/latlong.html>).

Nasal swabs

A nasal swab was obtained from all live and dead animals examined, using a nylon flocked swab, placed into universal transport medium (UTM) designed for room temperature storage and shipping (Swab and UTM: Catalogue no. 346C, Sterilin, Newport, UK). Swabs were

initially stored at 4°C and frozen at -80°C within 12 hours of collection until required.

Processing of the nasal swabs is detailed in Chapter 6.

Rectal swabs

Two rectal swabs were obtained from all live and dead animals examined, placed into Amies medium with charcoal (Medical Wire & Equipment, Corsham, UK) and stored at 4°C.

One rectal swab was processed for isolation of *Campylobacter* spp. bacteria as follows: In the field laboratory, the rectal swab was streaked onto *Campylobacter* charcoal differential agar (CCDA) plates (Oxoid, Basingstoke, UK) within 12 hours of sampling. The swab was subsequently frozen at -80°C, indefinitely, for future studies. Swabs taken from stranded grey seal pups presented to the Scottish SPCA National Wildlife Rescue Centre were sent by first class post to SAC Consulting Veterinary Services, Inverness, where they were streaked onto CCDA agar plates, incurring a delay of up to 5 days between sampling and processing in some cases (average: 2.35 days; Minimum 1 day; maximum 5 days).

The other rectal swab was processed for isolation of *Salmonella* spp. bacteria: In both the field laboratory and at Moredun Research Institute, the tip of the swab was aseptically severed and incubated for 24h at 37°C in selenite F broth (Oxoid) *Salmonella* enrichment medium.

Processing of the rectal swabs is detailed in Chapters 4 and 5.

Blood samples

Blood samples were obtained from the 90 live grey seal pups and 19 grey seal yearlings on the Isle of May. Blood was collected from the extradural vein using heparinised Vacutainer tubes (Becton Dickinson, Oxford, UK), stored at 4°C and processed within 12 hours. Tubes were spun at 2000xg for 10 minutes at room temperature (RT); plasma was collected and immediately stored in 1.8ml Nunc cryovials at -80°C (Thermo Fisher Scientific). The buffy coat was carefully removed from each sample; erythrocytes were lysed twice with a solution of Tris Ammonium Chloride pH7.2 and the resultant pellet washed and resuspended in phosphate

buffered saline (PBS) before being frozen at -80°C in 1.8ml cryovials. Subsequent processing of buffy coat samples is described in Chapter 6.

Dead pups

Post-mortem examination

A unique identifier was attributed to each animal as described above for the live pups and the following data were recorded: Sex, pup stage, mass to the nearest 100g; length (nose to tail) to the nearest 5mm, girth immediately posterior to the axilla (to the nearest 5mm); the presence of any external anomalies (bites, areas of alopecia, nasal discharge, ocular discharge) and the presence/absence of umbilical cord.

Necropsies were performed following a standard post-mortem procedure adapted from the “Guidelines for the post-mortem and tissue sampling of seals” by Kuiken and Baker (Kuiken & Baker 1991). An example of a gross post-mortem report and sampling collection checklist is provided in Appendix 1. Carcass condition was determined using the condition code 2 to 5 adapted from this protocol: condition code 2a: Freshly dead animal; condition code 2b: Subtle post-mortem change, haeme imbibition, slight bloating; condition code 3: moderate decomposition: moderate bloating, skin peeling, organs intact; condition code 4: advanced decomposition: marked bloating, skin peeling; condition code 5: indeterminate: mummified or skeletal remains. Body condition was determined taking into account sternal blubber depth and relative protuberance of the pelvis, rib-cage and neck and categorised as follows: overweight, fat, adequate, thin or emaciated.

A nasal swab for virology and two rectal swabs were collected from each animal as described above. Thirty-six tissues for histological examination, listed in Table 2-1, were stored in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with haematoxylin and eosin (H&E) for initial examination by light microscopy. Eyes were post-

fixed in modified Davidson's fixative (Latendresse et al. 2002) for 24h prior to processing. After initial examination, selected tissue sections were also stained with periodic acid Schiff (PAS), Gram, Ziehl-Neelson (ZN), Warthin-Starry silver and Congo red stains (Bancroft & Gamble 2008). Sections were observed using an Olympus BX50 microscope and photographed using an Olympus U-CMAD digital camera and AnalySIS Five software (Soft Imaging System GmbH, Münster, Germany).

Bacteriology

Samples for bacteriology were systematically and aseptically (see below) collected from brain, liver, lung and spleen and from any other lesions or exudate and were stored at -80°C. Subsequent processing of samples is described in Chapter 3.

Virology

Individual, approximately 5 x 5 x 5mm samples of 11 organs (liver, spleen, mesenteric lymph node, ileo-caeco-colic junction, kidney, left cranial lung lobe, bronchial lymph node, right ventricle, tonsil, brain and trigeminal ganglion, listed in Table 2-1) were collected aseptically, placed in sterile 1.8ml Nunc cryotubes (Thermo Fisher Scientific, Loughborough, UK) containing 1ml of sterile viral transport medium (Hanks balanced salt solution containing 0.02% phenol red, 1% w/v bovine albumin, 0.45 w/v sodium hydrogen carbonate, 600 µg/ml benzyl penicillin, 0.3 mg/ml streptomycin sulphate, 50 µg/ml polymyxin B and 50 µg/ml nystatin) and frozen at -80°C until required. Subsequent processing of samples is described in Chapter 6.

Chapter 2 – Sites and Sampling

Table 2-1 Routinely sampled tissues, fixation and intended application

Tissue sample	Fixation				
	Neutral Buffered Formalin	Frozen			
		-80°C	-80°C	-20°C	-80°C
Histopathology	Virology	Bacteriology	“Protozoal parasite screen”	“Viral tissue pool”	
Abdominal cavity					
Liver	X	X	X		X
Spleen	X	X	X		X
Mesenteric lymph node	X	X			X
Pancreas	X				
Stomach	X				
Small intestine	X				
Ileo-caeco-colic junction	X	X			X
Large intestine	X				
Kidney	X	X			X
Adrenal gland	X				
Urinary bladder	X				
Genital mucosa	X				
Testis/ Ovary	X				
Thoracic cavity					
Left cranial lung lobe	X	X	X	X	X
Right caudal lung lobe	X				
Bronchial lymph node	X	X			X
Trachea (mid)	X				
Diaphragm	X				
Right ventricle	X	X		X	X
Left ventricle	X				
Thymus	X				
Oesophagus (mid)	X				
Head and neck					
Thyroid gland	X				
Tonsil	X	X			X
Tongue (mid body)	X			X	
Buccal mucosa - cheek	X				
Skin - cheek	X				
Salivary gland	X				
Retropharyngeal lymph node	X				
Nervous system					
Brain	X	X	X	X	X
Pituitary gland	X				
Trigeminal ganglion	X	X			
Cervicothoracic ganglion	X				
Spinal ganglion	X				
Additional samples					
Bone marrow (10 th left rib)	X				
Eye (Right if present)	X				
Blood plasma		X			
Buffy coat		X			
Urine		X			

Pooled tissue samples for viral PCRs (viral tissue pools)

Pooled samples of 10 organs (listed in Table 2-1): liver, spleen, mesenteric lymph node, ileo-caeco-colic junction, kidney, left cranial lung lobe, bronchial lymph node, right ventricle, tonsil and brain were collected aseptically, placed in a sterile gentleMACS™ M tube (Miltenyi Biotec, Bisley, UK) and frozen at -80°C until required. These pooled tissue samples are subsequently referred to as “viral tissue pools”. Subsequent processing of samples is described in Chapter 6.

Pooled tissue samples for *T. gondii* and *N. caninum* PCRs (protozoal parasite screen)

Pooled samples of lung, tongue, heart and brain-stem were placed in a sterile tube and frozen at -20°C until required. These small pooled tissue samples are subsequently referred to as “protozoal tissue pools”. Subsequent processing of samples is described in Chapter 6.

Asepsis and disinfection

Asepsis between samples was achieved by using a Fireboy safety Bunsen burner with automated ignition (Intergra Biosciences AG, Zizers, Switzerland). Metal forceps, scissors and scalpel blades/holders were dipped into a solution of industrial methylated spirits (denatured alcohol) 99% v/v 74OP (Thermo Fisher Scientific, Loughborough, UK) and directly placed into the naked flame until red and left to cool between samples. Extensive disinfection of all equipment and the necropsy table was carried out between each animal using DuPont™ Virkon® S (Thermo Fisher Scientific) diluted 1:100 in fresh water.

Transport of samples from field laboratory

Movements of frozen samples between the field laboratory (Isle of May, Fife), Moredun Research Institute, Edinburgh and the SAC Consulting Veterinary Services, Inverness were carried out using large thermally insulated containers and dry ice. Due to logistical constraints, tissue samples collected prior to the 15th November (pups CD001-CD025) were transported for

approximately 1 hour using large thermally insulated containers and ice packs before being transferred to containers containing dry ice as above.

Statistical analysis

To evaluate the correlation between pup mass, pup stage and live/dead status, two-way analysis of variance (ANOVA) were performed with the glm procedure. The post-hoc Tukey's Honestly Significant Difference (HSD) test was used to calculate differences in mass between pup stages and live/dead status. To evaluate the relationship between yearling mass and sex, one-way analysis of variance (ANOVA) were performed with the glm procedure. Fisher's Exact Test (FET) was used to determine whether sex ratios were different between live and dead pups, separately for stranded and free-ranging pups.

Data were analysed using the R statistical software package (R Core Team 2013) and statistical significance was set at a p value of ≤ 0.05 .

2.4 Results

Sample numbers

Table 2-2 Number of grey seal pup samples in each sample group and per time point

Time	Stranded		Free-ranging				
	Live Pups	Dead Pups	Live Pups			Dead Pups	Live Yearlings
			Boulder beach	Muddy slope	Rocky pools		
Early	9	3	10	10	10	14	7
Mid	6	1	10	10	10	20	4
Late	9	4	10	10	10	16	8
Other	9	1	-	-	-	-	-
Total	33	9	30	30	30	50	19

A total of 90 live pups were sampled from 3 sites on the Isle of May at 3 different time points (Table 2-2). Although no pup production estimates were carried out on the Isle of May in 2011, this represents approximately 4.1% of the estimated 2010 birth cohort on the Isle of May.

A total of 50 dead pups were collected on the Isle of May, representing approximately 2.3% of the estimated 2010 birth cohort on the Isle of May and 21.4% of the overall mortality count (based on land based counts of dead pup carried out in 2012).

The 19 live yearlings were generally located at the periphery of the colony (Figure 2-5). Thirty three stranded live grey seal pups were sampled on arrival at the Scottish SPCA National Wildlife Rescue Centre from the locations specified in Figure 2-10.

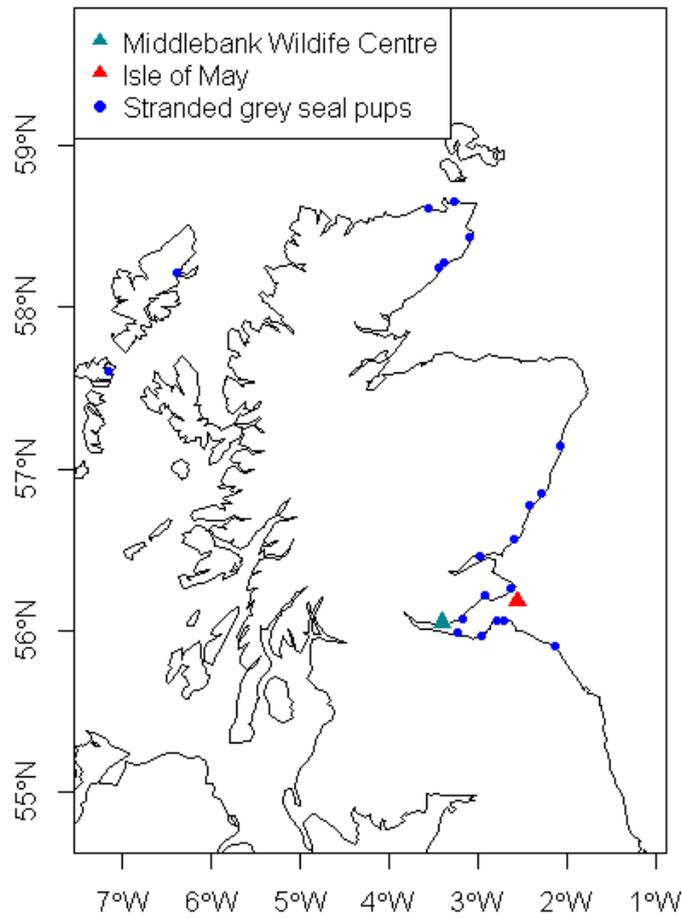


Figure 2-10 Map detailing stranding locations of grey seal pups submitted to the Scottish SPCA National Wildlife Rescue Centre

2.4.1 Distribution of pup and yearling mass

- Free-ranging pups and yearlings

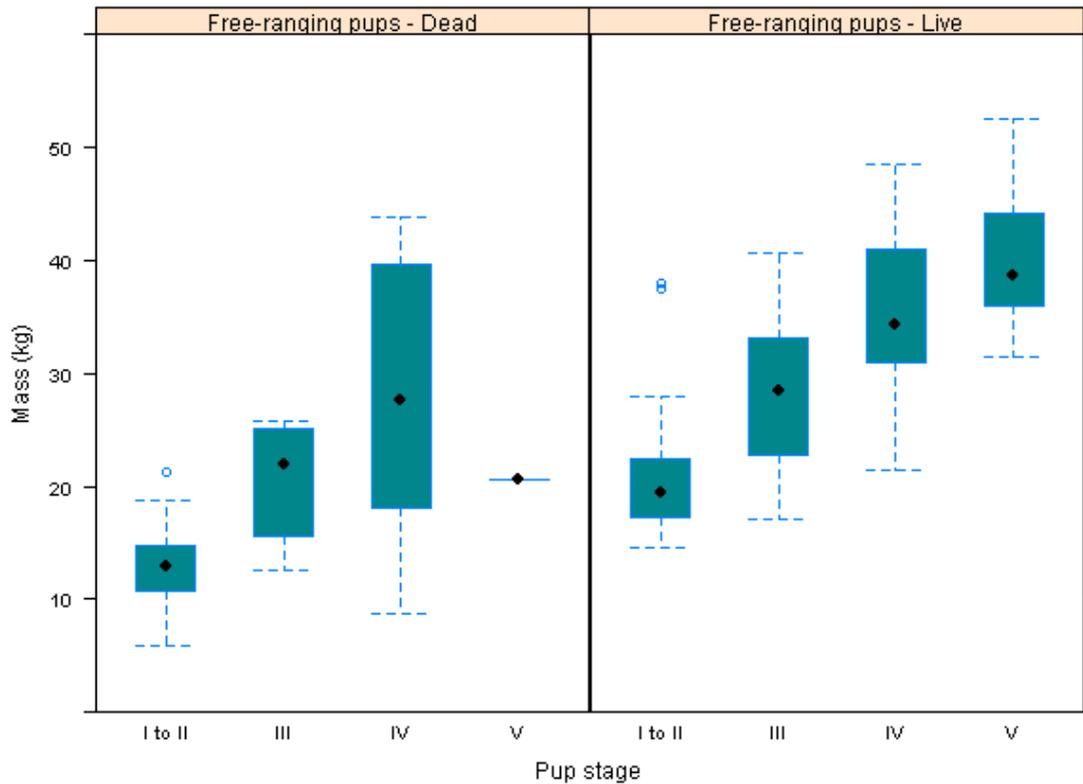


Figure 2-11 Mass distribution of dead and live free-ranging grey seal pups sampled on the Isle of May by pup stage

A two-way analysis of variance (ANOVA) on pup mass showed significant variation among pup stages and live/dead status, $F_{(4, 135)} = 57.6$, $p < 2.2e-16$, with dead grey seal pups weighing on average 10.96kg less than live grey seal pups. Post-hoc Tukey's HSD tests showed that the mass of pups in all stage groups were statistically significantly higher than the preceding stage with the exception of pup mass in pup stages IV and V groups which did not differ significantly given the small number of pups at stage V ($p=0.47$) (Table 2-3).

Table 2-3 Tukey post-hoc comparisons of pup mass within the Isle of May pups controlling for “Live/dead” status and pup stage (Stage I and II combined; Stage III; Stage IV and Stage V). Sign: Statistical significance. NS: Not significant; **: moderately significant; *** highly significant

Model parameter	Mean Difference	95% Confidence interval	Adjusted P-value	Sign.
Dead to Live	10.959	8.669, 13.249	<0.001	***
Stage				
Stage III to I-II	7.304	3.075, 11.534	<0.001	***
Stage IV to I-II	13.405	9.927, 16.883	<0.001	***
Stage V to I-II	16.821	11.062, 22.580	<0.001	***
Stage IV to III	6.100	1.411, 10.790	0.005	**
Stage V to III	9.517	2.955, 16.079	0.001	**
Stage V to IV	3.416	-2.689, 9.521	0.47	NS

Length, mass, girth and pup stage were highly correlated with each other (Figure 2-11 and Figure 2-12). So as not to violate the assumption in generalised linear models concerning the independence of independent variables, a single independent variable should be chosen to represent these four variables. Girth was proposed as a good indicator of body condition in weaned and post-weaned phocid seal pups (Hall and McConnell 2007), however this does not necessarily hold true for pre-weaned pups. Mass was therefore chosen as the most reliable, independently verified and reproducible of the morphometric measurements in the present study and retained for further analyses in subsequent chapters.

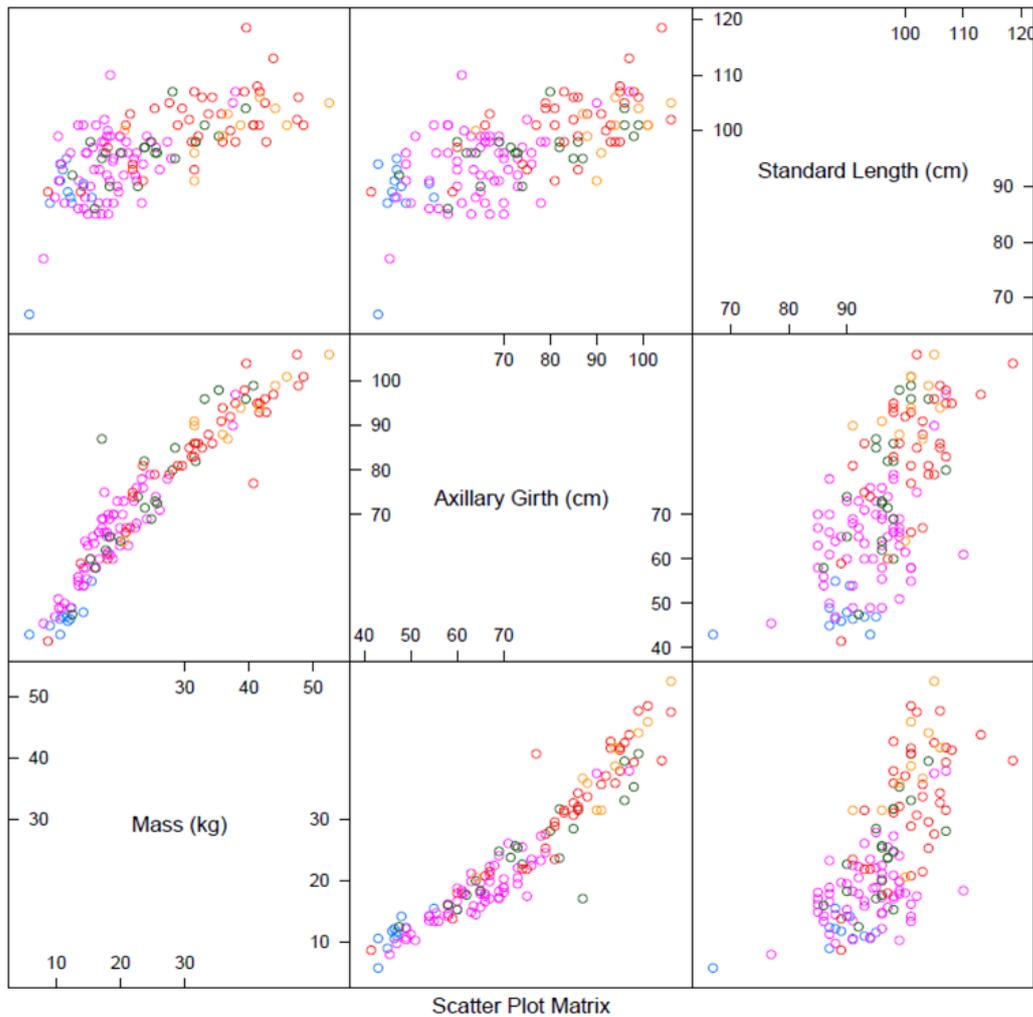


Figure 2-12 Scatterplot matrix showing positive correlation between girth, length and mass measurements in dead and live free-ranging grey seal pups. Colour of dot represents pup stage as specified in legend.

Mass distribution of live grey seal pups per sampling site

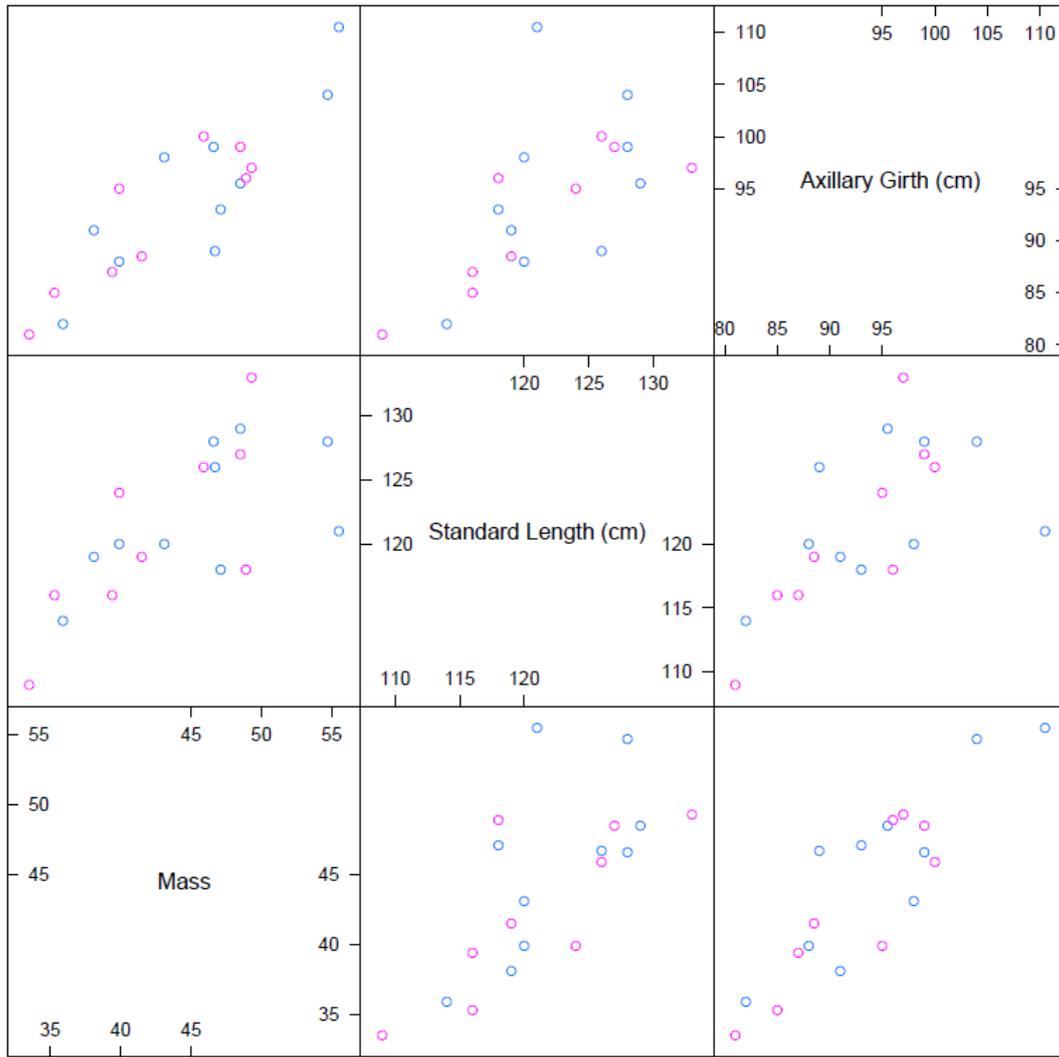
A one-way analysis of variance (ANOVA) on pup mass showed significant variation among sampling sites and pup stage, $F_{(2, 87)} = 3.5428$, $p = 0.033$), with live grey seal pups weighing on average 6.23kg and 4.88kg more on the rocky pool site than on the tidal boulder beach and the muddy, grassy slope, respectively. When correcting for pup stage, a two-way ANOVA on pup mass with post-hoc Tukey's HSD tests confirmed these differences (adjusted $p = 0.0128$ and $p = 0.002$) (Table 2-4)

Table 2-4 Tukey post-hoc comparisons of pup mass within live Isle of May pups controlling site and pup stage (Stage I and II combined; Stage III; Stage IV and Stage V). Sign: Statistical significance. NS: Not significant; **: moderately significant; *** highly significant

Model parameter	Mean Difference	95% Confidence interval	Adjusted P-value	Sign.
Site:				
Tidal boulder beach to rocky pools	-6.230	-10.240, -2.219	0.001	**
Muddy grassy slope to rocky pools	-4.883	-8.894, -0.873	0.013	*
Muddy grassy slope to tidal boulder beach	1.347	-2.664, 5.357	0.703	NS
Stage				
Stage III to I-II	7.864	2.432, 13.296	0.002	**
Stage IV to I-II	13.380	9.150, 17.609	<0.001	***
Stage V to I-II	17.577	11.296, 23.859	<0.001	***
Stage IV to III	5.516	-0.245, 11.276	0.06	NS
Stage V to III	9.713	2.314, 17.113	0.005	**
Stage V to IV	4.197	-2.371, 10.766	0.34	NS

- **Free-ranging yearlings**

The mean mass of live grey seal yearlings was 44.1 ± 6.30 kg. The mean mass of female yearlings was 42.47 ± 5.97 kg and the mean mass of male yearlings was 45.61 ± 6.52 kg Figure 2-14. This difference was not statistically significant (One-way ANOVA, $p=0.29$). Length, mass and girth were correlated with each other (Figure 2-13).



○ Male ○ Female

Figure 2-13 Scatterplot matrix showing positive correlation between girth, length and mass measurements in 19 live free-ranging grey seal yearlings. Colour represents sex of the animal: Blue: Male; Pink: Female.

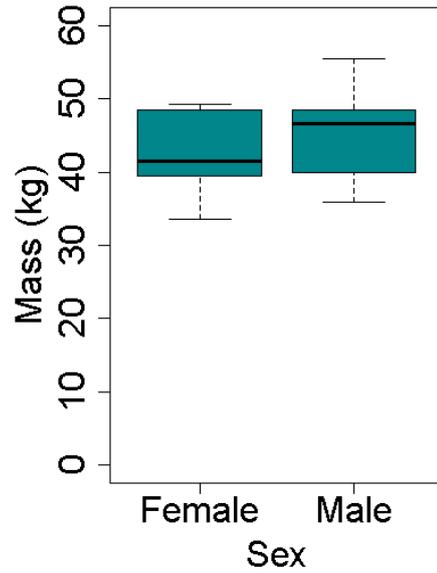


Figure 2-14 Mass distribution of live free-ranging yearlings sampled on the Isle of May by sex

- **Stranded grey seal pups**

There was no indication of an association between mass and pup stage or mass and dead/live status in stranded grey seal pups (Figure 2-15).

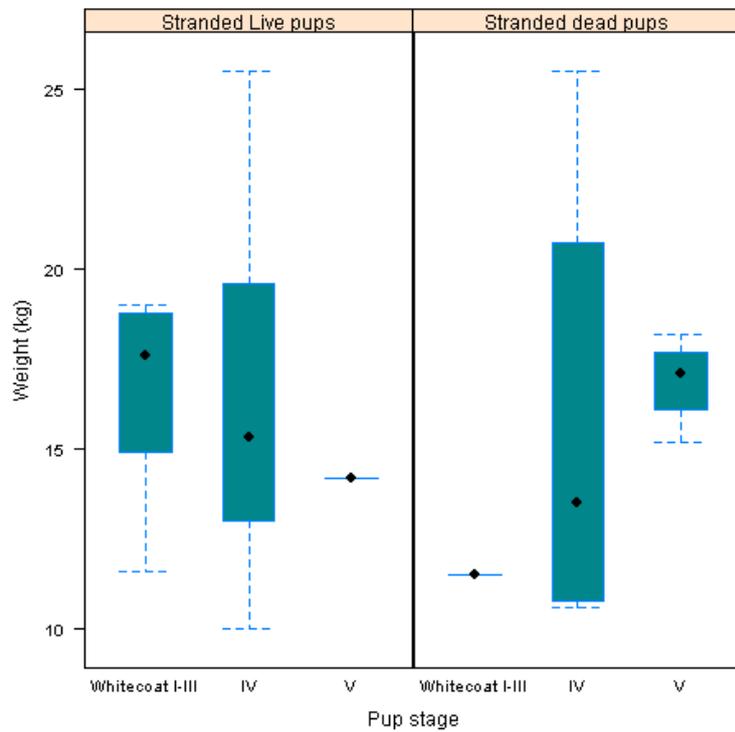


Figure 2-15 Mass distribution of stranded grey seal pups by pup stage and live/dead status

2.4.2 Sex distribution of pups sampled

The sex distribution of live grey seal pups and yearlings approached a 1:1 ratio. Male pups were however over-represented in both groups of dead pups (stranded (66.7%) and free-ranging (58%)) but this difference was not statistically significant ($p=0.3784$ and $p=0.4761$ for free-ranging and stranded pups, respectively. FET).

Table 2-5 Sex distribution of the grey seal pups and yearlings sampled for the study. * The sex of one pup in each of these two groups was not recorded

Sex	<u>Yearlings</u>	<u>Pups</u>				
	<u>Isle of May</u>	<u>All pups</u>	<u>Free-ranging</u>		<u>Stranded</u>	
	<u>Live (n=19)</u>	<u>(n=179)</u>	<u>Dead (n=50)</u>	<u>Live (n=89*)</u>	<u>Dead (n=9)</u>	<u>Live (n=31*)</u>
Male	10 (52.6%)	95 (53.1%)	29 (58%)	44 (49.4%)	6 (66.7%)	16 (51.6%)
Female	9 (47.4%)	84 (46.9%)	21 (42%)	45 (50.6%)	3 (33.3%)	15 (48.4%)

2.5 Discussion

A wide range and large number of samples were collected from free ranging grey seal pups and yearlings on the Isle of May, as planned. Disturbance to the colony was minimal and samples were processed promptly in a well equipped field laboratory. Sampling freshly dead carcasses, combined with rigorous aseptic technique and optimal cold storage facilities enabled collection of high quality samples for downstream processing.

The higher mass of pups on the rocky pool site, when compared to pups on the muddy, grassy slope, after controlling for pup stage, suggests that this site is the more favourable of the three for pup growth. The proximity of fresh water pools, and thus less need for females to spend time and energy commuting to water, may lead to increased time spent nursing the pup (Redman et al. 2001; Twiss et al. 2000).

Chapter 2 – Sites and Sampling

Despite the best efforts of the Scottish SPCA National Wildlife Rescue Centre, limited time and staffing constraints meant that the number of stranded grey seal pup samples submitted to the study was lower than expected, in particular the number of dead pups (n=9). The intention at the outset was to sample successfully rehabilitated grey seal pups at (or close to) release in order to compare pathogen carriage before and after rehabilitation. Sample kits and forms had been prepared to this effect but time and staffing constraints, along with constraints due to the building and preparation for the centre relocation to Fishcross in Clackmannanshire during the end of the 2011/2012 grey seal breeding season, prevented completion of this part of the study.

In hindsight, sampling of stranded grey seal pups during the 2012 pupping season rather than the 2011 season may have been a more realistic prospect from a logistical perspective, given the concurrent timing of the sampling on the Isle of May and at the rehabilitation centre. However, it seemed preferable to sample free-ranging and stranded grey seal pups simultaneously (during the same pupping season) so as to limit the effect of “year” on results. Indeed, delaying sampling of stranded seal pups until the following year may well have introduced substantial sampling bias. In addition, time constraints prevented this approach in the current study.

Despite the limitations, it was possible to make some broad comparisons between the data and results from the Isle of May pups with those arising from the rehabilitation centre.

Chapter 3 Neonatal mortality: Post-mortem findings in stranded and free-ranging grey seal pups

3.1 Introduction

The first studies examining the causes of mortality of grey seals were reported in the late 70's and 80's (Anderson et al. 1979; Baker 1980; Baker et al. 1980; Baker 1984; Baker 1987; Baker 1988; Baker & Baker 1988). These studies focused on pups, subadults and adults within their natal colonies. As discussed in Chapter 2, the UK grey seal population has doubled since these leading studies were conducted, from an estimated 44,700 in 1984 to 93,300 in 2011 (SCOS, 2012), leading to substantial changes in breeding colony dynamics. In particular, the number of pups born on the Isle of May, the most intensively studied grey seal breeding site in the UK, has doubled from approximately 1000 pups born annually in 1986, when the last detailed study of neonatal mortality study was carried out (Baker & Baker 1988), to an estimated 2300 in 2012 (SMRU, pers. comm.). Instead of leading to an increase in density of breeding females in pre-existing sites, areas of the island previously unutilised by females have become colonised and subsequently become densely populated pupping grounds (Pomeroy et al. 2000a). How this population increase has affected causes of pup mortality on this colony has not, to date, been studied.

The most frequently reported causes of death in neonatal grey seals include starvation, umbilical infection, other infections and trauma, present in variable proportions between colonies studied (Baker 1984; Baker 1988; Baker & Baker 1988). Two separate studies carried out on the Isle of May and North Rona, Scotland suggested that there were spatial and temporal differences in the causes of mortality within a grey seal breeding colony (Baker 1984; Baker & Baker 1988).

Since these early studies, the large majority of reports of disease in this grey seals has been obtained through examination of stranded animals (Alonso-Farre et al. 2011; Baker et al. 1995; Baker et al. 1998; Barnett & Westcott 2001; Barnett et al. 2000; Osterhaus et al. 1994), leading to a lack of current baseline health data on this species under natural conditions on their natal colonies.

The aims of this study were to investigate the causes of mortality in grey seal pups on the Isle of May during a single pupping season (2011) and to compare them with previously reported causes of mortality on this same colony established approximately 30 years ago (Baker & Baker 1988). A pathology based prospective study of the causes of mortality in grey seal pups was performed in parallel with screening for a large range of known and unknown viral, protozoal (see Chapter 6) and bacterial pathogens. A second study was performed over the same time frame investigating the causes of mortality and pathology present in stranded grey seal pups submitted to a wildlife rehabilitation centre (Scottish SPCA National Wildlife Rescue Centre, Middlebank Farm, Fife, Scotland) which subsequently died or were euthanised on welfare grounds.

Comparing causes of mortality on a colony and at a rehabilitation centre will help determine whether those described in stranded animals reflect the trends in their natal breeding colonies or whether they represent a biased subset of animals within the population.

A limitation of many published studies of morbidity and mortality in pinnipeds is the over-reliance on serological (Cabezón et al. 2011; Cornwell et al. 1992) or molecular techniques without correlating the findings with gross or microscopical lesions. Thus, the true pathological significance of many infectious agents remains unknown. It is likely that the presence of specific pathogens will be positively correlated with the presence of specific macro and microscopic lesions. To investigate this hypothesis, post-mortem examinations of dead seal

pups on the Isle of May were performed and samples taken for extensive pathogen screening. This work will further the understanding of the pathogenesis and significance of individual infectious agents in grey seal populations and potentially highlight interactions between different pathogens. The occurrence of bacterial flora will be compared with the historical published data by Baker and Baker (Baker & Baker 1988) wherever possible.

3.2 Materials and methods

3.2.1 Animals

Between 30th October and 2nd December, during the 2011 grey seal pupping season on the Isle of May, 50 freshly dead grey seal pups that had died without human intervention were subjected to extensive post-mortem examination. In parallel, 9 stranded grey seal pups, which had either died or were euthanised on humane grounds at the Scottish SPCA National Wildlife Rescue Centre, were also subjected to extensive post-mortem examination (see Chapter 2 for details).

3.2.2 Necropsy

Necropsies were performed as described in Chapter 2. A unique identifier was attributed to each animal and the following data recorded: date, location, sex, pup stage, mass to the nearest 100g; length (snout to tail) to the nearest 5mm, girth immediately posterior to the fore flippers; state of post-mortem preservation, nutritional condition, the presence of any external anomalies (e.g. bites, areas of alopecia, nasal discharge, ocular discharge) and the presence/absence of an umbilical cord.

Classification of major categories of mortality

Given the complexity of biological systems, it was often difficult to assign a single cause of death to each animal. An ultimate cause of death was assigned to each case, along with a list of

secondary lesions or findings for completeness. The proximate cause of death was determined as the event which was closest to, or immediately responsible for causing the death of the animal, such as trauma or euthanasia. This was established in contrast to a higher-level ultimate cause of death, considered the underlying reason(s) for the death of the animal, such as starvation or umbilical infection (omphalitis).

The ultimate cause of death was designated as one of the following categories, defined below: **‘stillbirth’**, **‘trauma’**, **‘starvation’**, **‘omphalitis’** and **‘septicaemia’**. Any one pup may have had a combination of the above conditions, but conditions were ranked in order of significance with respect to the cause of death. Finally, the ultimate cause of death was regarded as open or **‘miscellaneous’** where no clear cause of death could be determined.

‘Stillbirth’ was recognised by the presence of a wet, fresh umbilicus; wet pelage and often the presence of foetal membranes in close proximity to the carcass; **‘Starvation’** was identified by an extremely poor body condition with lack of blubber layer in non-perinatal pups.

‘Omphalitis’ was characterised by the simultaneous presence of suppurative omphalitis and severe peritonitis, often with copious abdominal effusion, the so-called “peritonitis-navel ill complex” described by Baker (Baker et al. 1980). **‘Septicaemia’** was determined by any or all of the following: suppurative pleuritis, peritonitis or meningitis associated with the isolation of bacteria from two or more visceral organs (other than that deemed to originate from omphalitis). A diagnosis of **‘trauma’** was attributed to pups with extensive subcutaneous haemorrhages in the absence of any other significant pathology.

Histopathology was performed routinely on 36 tissues and any specific lesions for each animal. All histopathology was read by a single pathologist (JB) and findings recorded in a Microsoft Access database. The following details were recorded for each lesion: organ; gross location;

histological location; process; severity; distribution; cell type or agent. In the absence of a tissue (due to processing or sampling error), the tissue was recorded as missing.

Immunohistochemistry targeting *Listeria monocytogenes* and *Pasteurella multocida* was performed on selected tissue sections. Endogenous peroxidase activity was blocked by immersion in 3% (vol/vol) H₂O₂-methanol for 20 min. Tissues were rinsed with running tap water for 5 min and incubated with 150 µl of 25% normal goat serum (NGS; Vector) in PBS-0.05% Tween 20 (PBST) for an hour at room temperature. Immunostaining was performed with Envision polymer reagents based on a standard avidin–biotin–peroxidase complex–based method, including reagents for *Pasteurella multocida* (polyclonal antiserum raised in a rabbit against *P. multocida* A:3 (Dagleish et al. 2010), dilution 1:2000) and *Listeria monocytogenes* (Rabbit anti *Listeria* O (DIFCO 223021) BD, Oxford, UK, monoclonal, dilution 1:4000) as primary antibodies. Details of incubation conditions, antigen retrieval and positive control samples used are summarised in Table 3-1.

Table 3-1 Antibodies and conditions used for immunohistochemistry

Pathogen targeted	Antibody (Ab) name, Source	Antigen retrieval	Primary Ab concentration	Secondary Ab	Positive control tissue
<i>Listeria monocytogenes</i>	Rabbit anti <i>Listeria</i> O (DIFCO 223021) (BD, Oxford, UK), monoclonal	0.1% trypsin and 0.1% calcium chloride in tris HCL pH7.8 for 50 minutes	1:4000 Overnight 4°C	Envision Goat anti rabbit HRP	Ovine cerebellum known to be infected with <i>L. monocytogenes</i>
<i>Pasteurella multocida</i>	<i>Pasteurella multocida</i> A:3 antibody 1 Rabbit polyclonal (Dagleish et al. 2010)	None	1:2000 Overnight 4°C	Envision Goat anti rabbit HRP	Bovine lung known to be infected with <i>P. multocida</i> A:3

3.2.3 Bacteriology

A single sample of liver, lung, spleen and brain were sampled aseptically from all seals submitted for post-mortem examination (see Chapter 2 for full details). Additional samples of

placenta (n= 2), larynx (n= 3), umbilicus (n= 8) and pus from a sub-mandibular abscess and a shoulder abscess were examined also. All tissue samples were frozen at -80°C at the time of post-mortem examination. The swab from the shoulder abscess was not frozen but was submitted directly to SAC Consulting Veterinary Services (SAC CVS), Inverness, UK. Routine diagnostic processing of bacteriological samples (Quinn et al. 2011b) was performed by Dr. Geoff Foster and Nick Davison at SAC CVS, Inverness. JB, under supervision from Dr. Geoff Foster, processed the bacteriological sample sets from 6 pups at SAC CVS, Inverness to be fully conversant with the techniques.

All tissues selected for bacteriological examination were thawed immediately prior to culture on Columbia agar with 5% sheep blood (CSBA) (Oxoid), MacConkey agar without salt¹ (Oxoid) and *Brucella*-specific Farrell's medium (Farrell 1974) (Oxoid). In addition, lung samples were cultured on Columbia chocolate agar with horse blood (CCHA) (Oxoid) and *Bordetella* selective medium (Oxoid). *Brucella*-specific Farrell's medium, CSBA and CCHA plates were incubated in a capnophilic atmosphere at 37°C. All remaining cultures were incubated aerobically at 37°C. MacConkey plates were examined for growth after 24 and 48 h; the *Bordetella* media was examined for up to 5 days and the Farrell's, CSBA and CCHA plates were examined daily for up to 14 days. Samples of placenta and any tissue submitted from suspected stillborn pups were also inoculated onto *Campylobacter* selective blood free (CCDA) agar (Oxoid) and incubated in a microaerophilic environment at 37°C for 96 hours. CCDA agar plates were examined for growth every 48h.

Resultant colonies were examined visually and sub-cultured onto CSBA plates prior to identification using routine bacteriological methods. All suspect *Mycoplasma* spp. isolates were

¹ The use of MacConkey agar without salt provides a 'low electrolyte medium' which markedly restricts the growth and spread of *Proteus* spp. a common contaminant which, due to its swarming growth pattern,

forwarded as agar blocks to the *Mycoplasma* Group at the Animal Health Veterinary Laboratories Agency (AHVLA, Weybridge, UK) for identification, as described by Foster (Foster et al. 2011). All suspect *Salmonella* spp. isolates were sent to Stobhill *Salmonella*, *Shigella* and *Clostridium difficile* reference laboratory (Glasgow, UK) for further identification (see Chapter 4).

Statistical analysis

Statistical analysis was performed using the R statistical software package (R Core Team 2013). For prevalence data, Fisher's exact test was performed. For each cause of mortality prevalence and odds ratios were calculated using a generalized linear model (GLM) with a binomial family and a logit link function. Sex, substrate, sampling time, adrenal mass, blubber thickness and presence/absence of lesions were used as fixed explanatory factors.

Sampling time ('early', 'mid', 'late', 'other') was inferred from date of sampling as described in Chapter 2. The substrate type ('grassy', 'boulder beach', 'rocky pools', 'other') was inferred from sampling location for free-ranging pups on the Isle of May as described in Chapter 2.

3.3 Results - Overview primary causes of mortality

3.3.1 Free-ranging grey seal pups - Isle of May - Ultimate cause of death

- **Overview**

In total, 50 pups were available for post-mortem examination on the Isle of May colony. Signalment, morphometric data, ultimate cause of death and secondary findings for each seal pup are presented in Table 3-2 and the most significant pathological findings are listed in Table 3-4.

When considering a single over-riding cause of mortality, a large majority of pups (78%, n = 39) died from either of two major causes: starvation and bacterial infection. A total of 15 (30%)

pups died from starvation, 13 (26%) from omphalitis –peritonitis complex and 11 (22%) from non-specific bacterial septicaemia. In addition 5 (10%) were stillborn or died due to dystocia, 2 (4%) died of primary trauma and a further 4 (8%) died of miscellaneous causes.

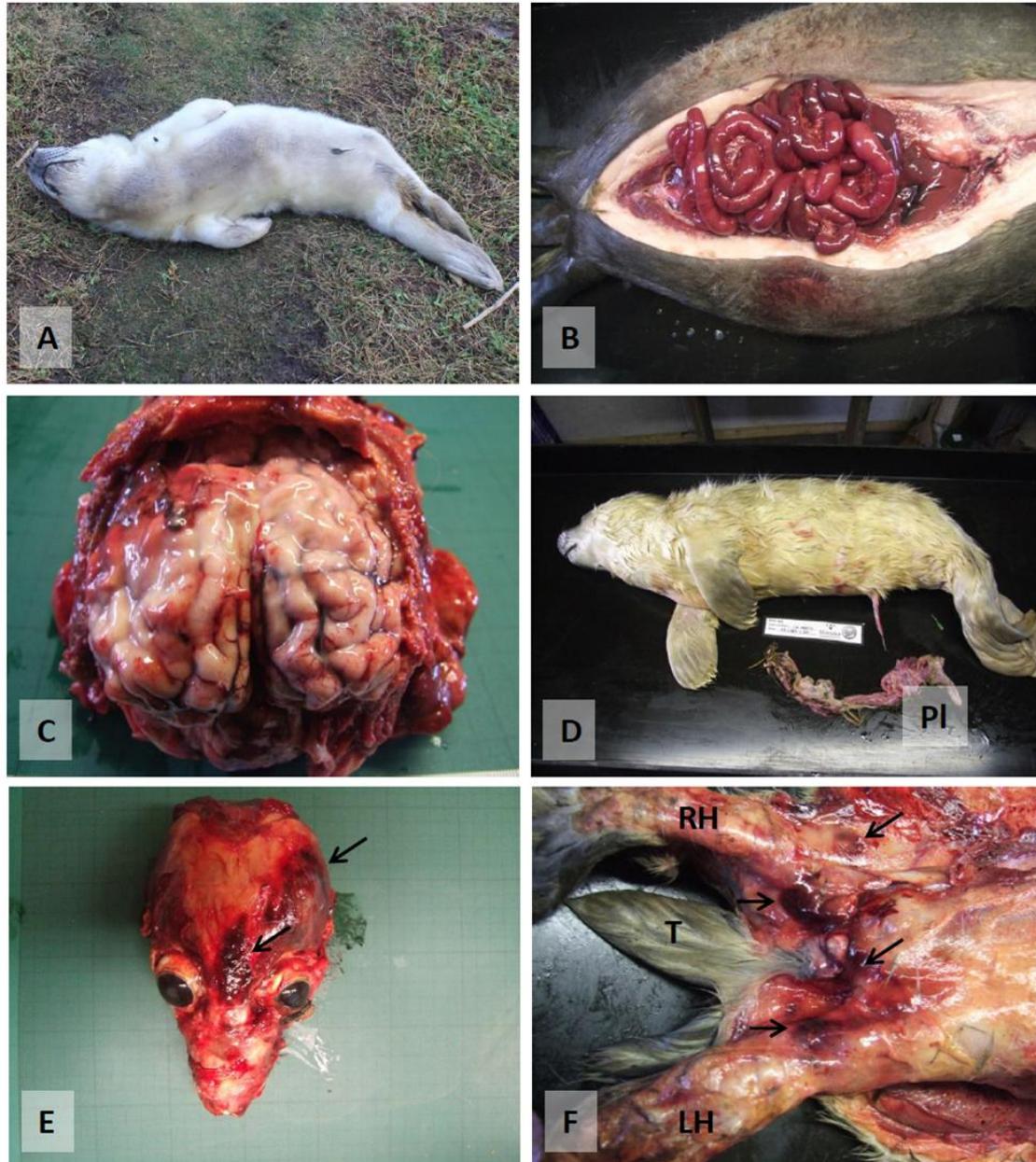


Figure 3-1 Principal categories of cause of death in grey seal pups A: Starvation. Live pup with prominent pelvis and costal arch; B: Omphalitis. Note excellent body condition of this pup, congested abdominal viscera and red soiling of the pelage surrounding the umbilicus (arrow); C: Septicaemia: Pup presenting with suppurative meningitis; D: Stillbirth. Note wet pelage, glistening pink, wet umbilicus and presence of placenta (PI.); E: Trauma. Suffusive haemorrhage within temporal musculature and dorsal and fronto-nasal suture (arrows). Note exophthalmos (right eye); F: Trauma. Undersize pup with multiple haemorrhages (arrows) within the perineum and pelvic region consistent with trauma and bite wounds. RH: Right hind limb; LH: Left hind limb; T: Tail.

Table 3-2 Summary of post-mortem findings for the 59 grey seal pups examined. Lgth: Length snout to tip of tail (cm); Blubber depth measured over sternum; Ultimate COD: Ultimate Cause of death; Ph-HV1: Result of PhHV1 PCR (combined result of nasal swab and pooled tissue sample (see Chapter 6)); Rectal Campy: Isolation of *Campylobacter* spp. from rectal swab (see Chapter 5); Rectal Salm: Isolation of *Salmonella* spp. from rectal swab (see Chapter 4); Thy Atr: Degree of thymic atrophy (0: none; 1: minimal; 2: mild; 3: moderate; 4: severe); UI: presence of umbilical infection (omphalitis); NA: not available

Seal	Sex	Mass (kg)	Lgth (cm)	Blubber depth (mm)	Pup Stage	Date	Ultimate COD	Overview and secondary findings	Ph-HV1	Rectal Campy	Rectal Salm	Thy Atr	UI
CD001	M	11	87	3	II	30/10/11	Starvation	Starvation; <i>E. coli</i> septicaemia; Interstitial pneumonia (<i>Streptococcus phocae</i> and <i>E. coli</i>); thyroid hyperplasia and mild omphalitis, random multifocal hepatitis (<i>E. coli</i>)	-	-	-	3	+
CD002	F	14	86	6	II	30/10/11	Septicaemia	Septicaemia; 4 bacteria in septicaemic distribution: <i>Streptococcus halichoeri</i> ; <i>S. phocae</i> ; <i>A. phocae</i> and <i>L. monocytogenes</i> ; Suppurative meningo-encephalitis; pulmonary oedema; omphalitis; vasculitis	+	-	-	3	+
CD003	M	21	96	1	II	31/10/11	Septicaemia	<i>E. coli</i> septicaemia; Starvation; interstitial pneumonia; suppurative meningo-encephalitis; adrenal necrosis	+	-	-	1	-
CD004	F	18	100	12	II	01/11/11	Trauma	Haemo-abdomen; Haemorrhages skull and brainstem	-	-	-	0	-
CD005	F	14	90	7	I	01/11/11	Stillborn	Dystocia; Placenta over nares; Bilateral exophthalmos and hyphaema; Suffusive haemorrhage and oedema neck and shoulder; Autolysis of organs caudal to shoulder	-	-	-	0	-
CD006	M	16	88	6	I	02/11/11	Stillborn	Dystocia; Bilateral exophthalmos; Haemorrhage/oedema masseters and temporal muscle; Pulmonary oedema and congestion	-	-	-	0	-
CD007	F	18	110	12	II	02/11/11	Omphalitis	Omphalitis; peritonitis; adrenal necrosis; suppurative meningitis with thrombosis and vasculitis. <i>Streptococcus phocae</i> (brain and umbilicus)	-	-	-	0	+
CD008	F	12	95	1	I	03/11/11	Starvation	Starvation; <i>Streptococcus dysgalactiae</i> septicaemia; peritonitis	-	+	-	2	+
CD009	M	11	93	1	I	03/11/11	Starvation	Starvation; Gastric infarcts; mild umbilical infection (<i>S. phocae</i> and <i>E. coli</i> isolated from urachus); renal mineralisation	-	+	-	1	+
CD010	M	14	101	6	II	05/11/11	Other	Abdominal and thoracic effusion; splenic infarction with helminth parasite profiles; gliosis; suspect cerebral infarction	-	-	-	0	-
CD011	M	15	96	9	II	06/11/11	Septicaemia	<i>E. coli</i> septicaemia	+	-	-	2	+
CD012	M	10	91	2	II	07/11/11	Starvation	Starvation; secondary trauma	+	+	-	2	-
CD013	M	10	99	2	II	08/11/11	Starvation	Starvation; <i>E. coli</i> septicaemia; omphalitis (<i>E. coli</i> and <i>S. phocae</i>); vasculitis	+	+	-	3	+
CD014	F	12	89	12	I	09/11/11	Stillborn	Dystocia; Bilateral hyphaema; Haemorrhages skull, left jaw and ribs; Pulmonary atelectasis	-	-	-	0	-

Chapter 3 – Neonatal pathology

Seal	Sex	Mass (kg)	Lgth (cm)	Blubber depth (mm)	Pup Stage	Date	Ultimate COD	Overview and secondary findings	Ph-HV1	Rectal Campy	Rectal Salm	Thy Atr	UI
CD015	M	14	91	3	II	10/11/11	Starvation	Starvation; Gastric infarcts; Multifocal hepatic necrosis with suppurative hepatitis; <i>E. coli</i> (lung); suspected <i>E. coli</i> septicaemia or Phocid Herpesvirus 1	+	-	+	2	-
CD016	F	15	96	23	II	11/11/11	Omphalitis	Omphalitis; Peritonitis; Moderate lymphoid atrophy; <i>S. equi</i> , <i>S. phocae</i> and <i>A. phocae</i> isolated from spleen, liver and lung	+	+	+	3	+
CD017	M	24	97	27	III	11/11/11	Septicaemia	Septicaemia originating from sub-mandibular cellulitis; Suppurative meningo-encephalitis; Myocarditis; Pneumonia; Gram negative non fermenter in septicaemic spread and <i>S. phocae</i> in liver	+	+	-	3	-
CD018	M	25	98	21	III	12/11/11	Septicaemia	<i>Pasteurella multocida</i> septicaemia; fasciitis possibly originating from bite wound	+	+	-	NA	-
CD019	M	9	87	10	I	13/11/11	Stillborn	Dystocia; Bilateral exophthalmos; Haemorrhage/oedema masseters and head, neck, cervical vertebrae	-	-	-	0	-
CD020	M	13	86	6	II	13/11/11	Starvation	Starvation; Trauma; <i>E. coli</i> and <i>L. monocytogenes</i> isolated	+	-	-	2	-
CD021	M	13	101	3	II	14/11/11	Starvation	Starvation; Large facial abscess leading to septicaemia (Gram positive rods and <i>E. coli</i> in lung); jaundice; hepatic necrosis	+	-	-	3	+
CD022	M	16	101	13	II	14/11/11	Omphalitis	Omphalitis; Peritonitis; Trauma (proximate); <i>A. Phocae</i> septicaemia secondary to umbilical infection	+	+	-	2	+
CD023	F	9.8	88	1	II	14/11/11	Starvation	Starvation; Omphalitis	+	-	-	3	+
CD024	F	12	96	9	II	15/11/11	Omphalitis	Omphalitis-Peritonitis-Hepatitis complex leading to septicaemia (<i>Streptococcus halichoeri</i> and <i>E. Coli</i> in liver; <i>L. monocytogenes</i> and <i>A. Phocae</i> in spleen); Colitis	+	+	-	3	+
CD025	F	18	97	15	IV	15/11/11	Omphalitis	Omphalitis-Peritonitis-Hepatitis complex; Gastritis; Colitis, Enteritis; Panophthalmitis; bites, Pulmonary oedema; <i>S. phocae</i> (liver/spleen); <i>L. monocytogenes</i> (spleen)	-	+	-	3	+
CD027	F	26	96	31	III	16/11/11	Omphalitis	Omphalitis-Peritonitis-Hepatitis complex; Moderate immunosuppression; Broncho-interstitial pneumonia (<i>P. multocida</i>); Ulceration larynx and epiglottis; <i>Mycoplasma</i> spp. (larynx); <i>S. agalactiae</i> ; <i>S. halichoeri</i> and <i>S. phocae</i> (liver, spleen and lung)	+	+	-	3	+
CD028	M	44	113	55	IV	16/11/11	Other	Abdominal effusion; pulmonary oedema; cerebral gliosis, Gitter cells and vacuolation; no bacterial growth; very good condition	-	+	+	0	-

Seal	Sex	Mass (kg)	Lgth (cm)	Blubber depth (mm)	Pup Stage	Date	Ultimate COD	Overview and secondary findings	Ph-HV1	Rectal Campy	Rectal Salm	Thy Atr	UI
CD029	M	16	86	21	III	17/11/11	Omphalitis	Omphalitis-Peritonitis-Hepatitis complex; Interstitial pneumonia; Laryngeal ulceration; <i>S. phocae</i> (liver, lung); <i>A. phocae</i> (Liver, spleen); <i>L. monocytogenes</i> (spleen)	+	+	-	0	+
CD030	M	15	98	12	III	17/11/11	Septicaemia	<i>Pasteurella multocida</i> septicaemia; Meningo-encephalitis; Fibrino-necrotising pleuro-pneumonia; Endocarditis; Peritonitis; Laryngeal ulceration; <i>Streptococcus canis</i> (spleen, liver and brain) and <i>S. agalactiae</i> , <i>A. phocae</i> and <i>Mycoplasma phocicerebrale</i> (Lung)	+	-	-	3	+
CD031	M	40	119	51	IV	17/11/11	Septicaemia	<i>Listeria monocytogenes</i> septicaemia; leuco-encephalitis with glial foci; Suppurative pleuritis, emphysema and oedema	-	+	+	3	-
CD032	M	11	94	1	II	18/11/11	Starvation	Starvation; Septicaemia (<i>A. phocae</i>); mild omphalitis (suspected portal of entry); fibrino-suppurative meningoencephalitis with vasculitis; gastric infarcts; Moderate chronic-active colitis	+	+	-	NA	+
CD033	F	25	96	21	III	18/11/11	Omphalitis	Omphalitis; Peritonitis; hepatitis; pneumonia and splenitis (<i>S. phocae</i> , <i>S. canis</i> , <i>S. zooepidemicus</i> and <i>A. phocae</i>); lymphohistiocytic meningo-encephalitis in brainstem with glial foci	+	+	+	1	+
CD034	F	42	101	56	IV	19/11/11	Other	Asymmetrical laryngeal necrosis with foreign body (plant material); Broncho-pneumonia and possible septicaemia (<i>Streptococcus phocae</i> ; <i>A. phocae</i> ; <i>E. coli</i>); fibrino-suppurative arthritis; Bite wounds	+	+	-	1	-
CD035	M	18	99	23	II	19/11/11	Septicaemia	<i>Listeria monocytogenes</i> septicaemia; (Lympho)histiocytic meningo-encephalitis, Fibrino-suppurative pneumonia, Suppurative splenitis and hepatitis; large bite wounds	+	-	-	3	-
CD036	M	19	95	25	II	21/11/11	Omphalitis	Omphalitis; peritonitis; Interstitial pneumonia; <i>A. phocae</i> ; <i>E. coli</i> ; <i>L. monocytogenes</i> ; <i>S. phocae</i>	+	+	+	3	+
CD037	F	28	105	33	IV	21/11/11	Omphalitis	Omphalitis; Peritonitis; Interstitial pneumonia	+	+	-	0	+
CD038	M	21	101	20	IV	22/11/11	Septicaemia	Septicaemia (<i>Pseudomonas aeruginosa</i>); suppurative meningo-encephalitis with vasculitis and necrosis; suppurative splenitis, colitis and massive aspiration pneumonia; <i>Toxoplasma</i> PCR positive	+	+	+	3	-
CD039	F	20	96	19	III	22/11/11	Trauma	Suffusive haemorrhage (temporal muscle; peri-ocular and ventral neck)	-	+	-	0	+

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Seal	Sex	Mass (kg)	Lgth (cm)	Blubber depth (mm)	Pup Stage	Date	Ultimate COD	Overview and secondary findings	Ph-HV1	Rectal Campy	Rectal Salm	Thy Atr	UI
CD040	F	11	91	1	I	23/11/11	Starvation	Starvation; Gastric ulceration; Atrophic enteritis and secondary trauma (cranium)	+	-	+	3	+
CD041	M	11	94	2	I	23/11/11	Starvation	Starvation; <i>Erysipelothrix rhusiopathiae septicaemia</i> ; Suppurative meningo-encephalitis with gliosis and vasculitis; Small intestinal vasculitis with necrosis of villi; Centrilobular hepatocellular necrosis; vasculitis; Moderate chronic-active pneumonia; <i>Listeria monocytogenes</i> (Spleen and Liver)	+	-	-	3	-
CD042	F	14	89	15	IV	23/11/11	Omphalitis	Omphalitis; Peritonitis; Hepatitis with mid-zonal hepatic necrosis; <i>Listeria monocytogenes septicaemia</i> ; vasculitis spleen; ulcerative cystitis; Pyogranulomatous interstitial pneumonia; Fibrino-suppurative uveitis; Granulomatous encephalitis with vasculitis, type II astrocytes and Gitter cells	+	-	-	3	+
CD043	F	14	91	6	I	23/11/11	Omphalitis	Omphalitis; Centrilobular and mid-zonal hepatic necrosis; <i>E. coli septicaemia</i> ; Mucosa associated lymphoid tissue necrosis; Pleuritis with pyogranulomatous and fibrinous pneumonia; Cerebral oedema with Gitter cells, Alzheimer type II astrocytes; Jaundice	-	-	-	1	+
CD044	F	21	100	20	V	24/11/11	Septicaemia	Septicaemia: <i>Salmonella Bovismorbificans</i> and <i>Streptococcus phocae</i> ; Mild omphalitis; Gastric erosion; Chronic-active interstitial pneumonia; Suppurative meningo-encephalitis, spinal abscess; dermal hyperkeratosis	+	-	+	3	+
CD045	M	12	88	12	I	24/11/11	Omphalitis	Omphalitis; Peritonitis; Septicaemia: <i>Salmonella Bovismorbificans</i> , <i>A. phocae</i> , <i>S. phocae</i> ; Pyogranulomatous and fibrinous bronchopneumonia; nasal mucosal ulceration; Multifocal random hepatic necrosis	+	-	+	3	+
CD046	F	5.8	67	7	I	26/11/11	Other	Undersize; Septicaemia: <i>Aeromonas hydrophila</i> ; Trauma; Bile stasis	-	+	-	1	-
CD047	M	8.7	89	1	IV	26/11/11	Starvation	Emaciation; Hyperthyroidism - goiter; Gastric and colonic ulceration; Cerebral white matter vacuolation; Gitter cells; Mild neuronal necrosis	+	-	-	3	+
CD048	M	13	92	8	III	28/11/11	Starvation	Emaciation; Septicaemia: <i>Salmonella Bovismorbificans</i> , <i>Actinomyces sp.</i> , <i>Streptococcus agalactiae</i> ; Gastric ulceration; Colitis; Omphalitis; peritonitis; Hepatitis; Moderate thyroid hyperplasia; Laryngeal necrosis; Lymphohistiocytic meningo-encephalitis with vasculitis	+	+	+	3	+

Seal	Sex	Mass (kg)	Lgth (cm)	Blubber depth (mm)	Pup Stage	Date	Ultimate COD	Overview and secondary findings	Ph-HV1	Rectal Campy	Rectal Salm	Thy Atr	UI
CD049	M	12	87	8	I	29/11/11	Stillborn	Stillborn; Pleural oedema; Intra-alveolar meconium (suspected asphyxiation on allantoic fluid)	-	-	-	0	-
CD050	F	8	77	2	II	01/12/11	Starvation	Emaciation; Hepatitis; Hepatic and adrenal necrosis; Atrophic and ulcerative enteritis and colitis associated with <i>Salmonella</i> Bovismorbificans; Endocarditis; Prominent vasculitis; <i>Pseudomonas aeruginosa</i> (septicaemic distribution); <i>Listeria monocytogenes</i> (Liver)	+	+	+	3	+
CD051	M	30	104	45	IV	02/12/11	Septicaemia	Septicaemia: <i>Salmonella</i> Typhimurium, <i>Listeria monocytogenes</i> ; Granulomatous meningo-encephalitis	+	-	+	0	-
R 001	M	26	89	47	IV	04/11/11	Other	Euthanasia - Bilateral ulcerative keratitis with uveitis; Pulmonary alveolar oedema	+	-	-	0	-
R 002	F	16	83	12	IV	04/11/11	Other	Euthanasia - Bilateral ulcerative keratitis	+	-	-	2	-
R 003	F	12	89	0	II	08/11/11	Starvation	Euthanasia; Emaciation; Suppurative arthritis and cellulitis (tibio-tarsal joint); Diarrhoea	+	-	-	3	-
R 004	M	15	97	4	V	16/11/11	Starvation	Emaciation; Vasculitis, DIC, peritonitis, aspiration milk; Bite wounds	+	-	-	3	-
R 005	M	18	93	24	V	24/11/11	Other	Euthanasia; Poxviral dermatitis	+	-	-	4	-
R 006	M	11	93	0	IV	24/11/11	Starvation	Emaciation; Hyperthyroidism; Metastatic mineralisation; Diarrhoea	+	+	-	NA	-
R 007	F	17	92	29	V	26/11/11	Septicaemia	Euthanasia; Septicaemia: <i>Pseudomonas aeruginosa</i> ; originating from shoulder abscess; Suppurative splenitis, meningitis, pneumonia	+	-	+	NA	-
R 008	M	17	89	8	V	02/12/11	Septicaemia	Euthanasia; Poor body condition; Septicaemia: <i>Pseudomonas aeruginosa</i> ; Pyogranulomatous meningo-encephalitis; Corneal ulceration; Vasculitis; Hepatic necrosis	+	-	-	3	-
R 009	M	11	83	0	IV	16/01/12	Starvation	Emaciation; Gastric ulceration	+	-	-	3	-

- **Ultimate cause of death**
 - **Starvation/malnutrition**

Starvation was the most common ultimate cause of death of pups on the Isle of May colony with 15 (30%) pups succumbing to starvation. The principal findings were that affected pups were significantly underweight with a thin to emaciated body condition. The mean blubber thickness was 2.5mm (95% CI: 1.5, 3.5mm) which was significantly lower than blubber thickness of pups dying of septicaemia (21.4mm; 95% CI: 12.12, 30.68), omphalitis (18.2mm; 95% CI: 13.6, 22.8) or of miscellaneous causes (31mm; 95% CI: 2.72, 59.28) ($p < 0.001$ in all 3 comparisons).

Gastric ulceration was a frequent feature in emaciated pups (40%, 6/15) and this was not present in any other group. Ulceration manifested as dark brown to black, well demarcated, irregularly shaped depressions in the gastric mucosa (Figure 3-2A), predominantly within the pyloric region. Histologically, ulcers were similar to those described by Baker et al. (1980) and consisted of a segmental area of full thickness mucosal necrosis, often with a sharp demarcation between affected and unaffected mucosa (Figure 3-4A). The submucosal vasculature underlying the lesion was frequently occluded by recent to organising fibrin thrombi (Figure 3-4B).

Additionally, the adrenal mass was significantly lower in pups dying from starvation than in pups dying from non-specific bacterial septicaemia ($p = 0.003$) or from the omphalitis-peritonitis complex ($p < 0.001$). Other characteristic findings in this group were soiling of the perineum (46.7%, 7/15) and a small thymus mass (mean 8.0g (95% CI: 5.4, 10.7)). Umbilical infection (omphalitis) was frequent in this group (73.3%, 11/15) and 20% (3/15) pups had grossly evident peritonitis. The stomach lumen was generally empty (66.7%, 10/15) and pica, represented by ingestion of plant material, small stones, seaweed or fur, was present in 20% (3/15) cases. Other findings included subjectively large thyroid glands (26.6%, 4/15) with histological evidence of mild thyroid hyperplasia in 20% (3/15) pups. Multifocal hepatic necrosis reminiscent of that seen with herpesvirus infection in harbour seals was noted in 53.3% (8/15) pups. Nasal mites

were seen in 33.3% (5/15) pups in this group. Within pups for which starvation was the ultimate cause of death, two individual animals presented with a septicaemic spread of *Erysipelothrix rhusiopathiae* and *Escherichia fergusonii*, respectively.

○ **Omphalitis-peritonitis complex**

The second most common ultimate cause of death was attributed to **omphalitis (umbilical infection)**, ultimately leading to peritonitis, hepatitis and septicaemia and affected 13/50 (26.0%) of the pups examined on the Isle of May. Omphalitis was recognised by tracking, liquid to inspissated, purulent material along the umbilical veins and arteries, frequently reaching the hepatic ductus venosus and portal vein (Figure 3-2C). Occasionally, the umbilicus was entirely replaced by necrotic tissue, leaving a direct communication between the peritoneum and the exterior of the animal, as described previously by Baker (1980) and serosanguinous to purulent abdominal effusion was common (69.2%, 9/13). The peritoneum was frequently dark red with florid inflammation of the mesentery (Figure 3-2D), contraction of small and large intestinal muscularis and serosa and extension of inflammation along the tunica vaginalis leading to peri-orchitis in 2 cases (50.0%, 2/4 males). Lesions suggestive of septicaemia such as pneumonia (8/13 pups (61.5%)), thoracic effusion (3/13, 23.0%), splenitis (5/13, 38.5%) and meningitis (2/13, 15.4%; lymphohistiocytic and pyogranulomatous – 1 case of each)) were also seen and the adrenal glands were significantly ($p < 0.001$) larger in this group (mean 4.18g (95% CI: 3.52, 4.85)) compared to pups dying of starvation (mean 2.84g (95% CI: 2.41, 3.27)). Finally, the body condition of these pups varied from thin to overweight.

Arcanobacterium phocae (7), *E. coli* (6), *Streptococcus phocae* (8), *Listeria monocytogenes* (5) and, in a single case, *Salmonella Bovismorbificans* (1) were associated with a diagnosis of omphalitis-septicaemia. Statistically, *Streptococcus phocae* ($p=0.011$ and $p=0.040$, FET) and *Arcanobacterium phocae* ($p=0.008$ and $p=0.003$, FET) were both positively associated with the presence of umbilical infection and peritonitis/serositis on histological examination, irrespective of ultimate cause of death. Furthermore, there was a positive association between the presence

of *Streptococcus phocae* and *Arcanobacterium phocae* (p=0.027, FET). The presence of either bacterium increases the chance of carrying the other by a factor of 5.25 (p=0.019, 95% CI: 1.31, 21.03, glm).

Table 3-3 Relationship between bacterial isolates and presence of omphalitis/peritonitis. Odds ratios, 95% confidence intervals and p-values of binomial generalised linear models

Bacterial species	Presence of omphalitis	Histological evidence of peritonitis/serositis
<i>Streptococcus phocae</i>	7.72 (1.51, 39.41) (p=0.014)	4.57 (1.23, 16.93) (p=0.023)
<i>Arcanobacterium phocae</i>	12.22 (1.43, 104.27) (p=0.022)	8.86 (2.07, 37.9) (p=0.003)

○ **Septicaemia**

Non specific septicaemia (not arising from omphalitis) was the ultimate cause of death of 11/50 (22.0%) of the dead pups examined on the Isle of May colony. Case signalment included the presence of a markedly congested subcutaneous vasculature (11/11, 100%), peripheral lymphadenopathy (8/11, 72.7%); congested peritoneum (5/11, 45.5%); consistently abnormal lungs on gross examination, of which 90.9% (10/11) had histological evidence of pulmonary interstitial oedema and 72.7% (8/11) mild to severe interstitial pneumonia. In addition, 18.2% (2/11) of pups presented with thoracic effusion; 27.2% (3/11) had grossly evident meningitis (Figure 3-2); 90.9% (10/11) histological meningitis and 81.8% (9/11) encephalitis. Four pups (36.4%, 4/11) had some degree of omphalitis although no gross or histological evidence of peritonitis was noted, differentiating this group from pups whose ultimate cause of death was attributed to omphalitis.

The majority (90.9%, 10/11) of pups in this group had skin lesions consistent with bites. In one case, the primary site of infection may have been a flipper tagging site and in another a puncture wound in the thorax was contiguous with extensive fasciitis over the right thorax and underlying fibrino-suppurative pleuro-pneumonia.

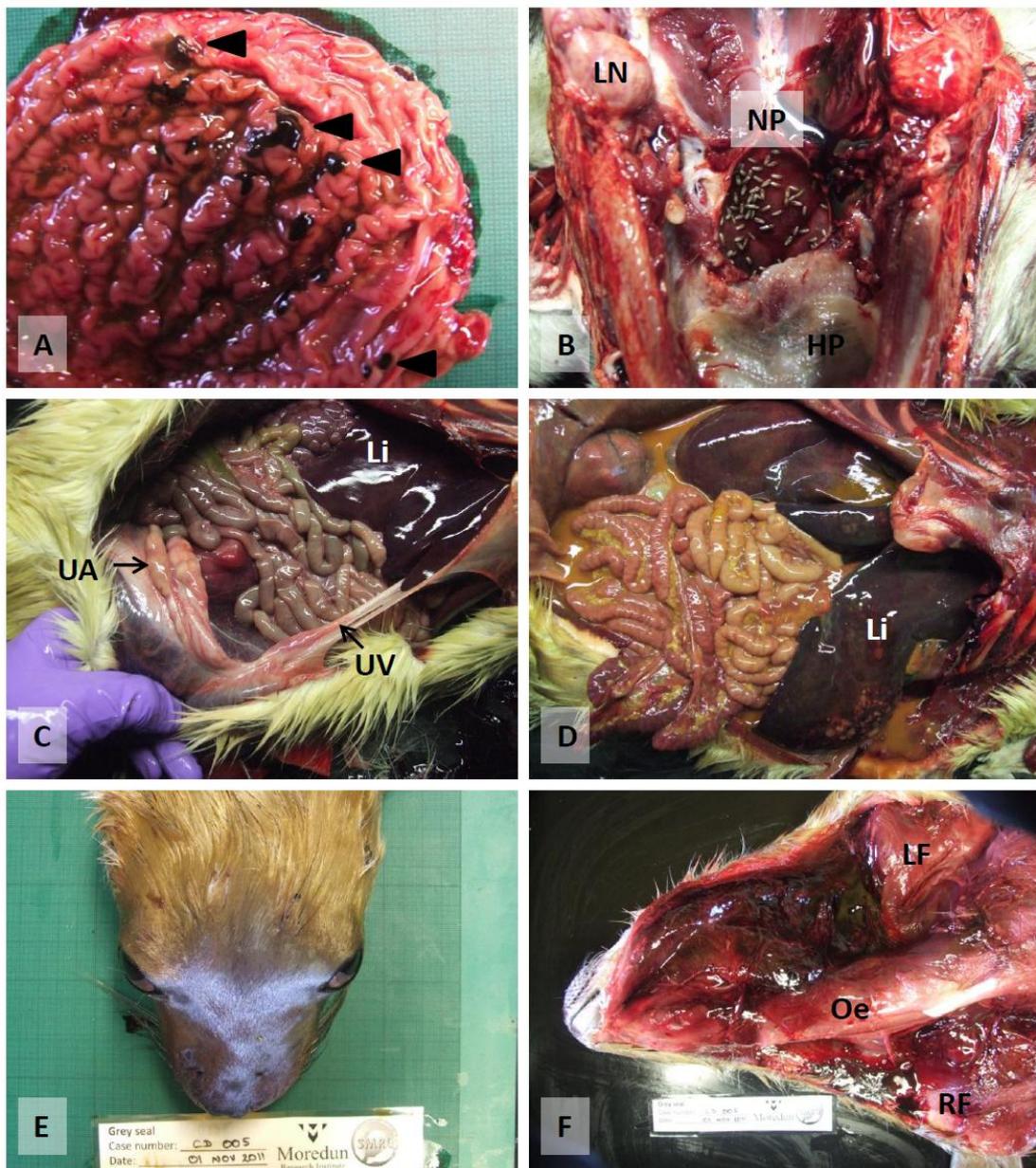


Figure 3-2 Gross pathology in grey seal pups sampled on the Isle of May. A. Multifocal gastric ulceration. Arrowheads indicate ulcers; B: Nasal mites in nasopharynx (NP) of a grey seal pup suffering from starvation. LN: Retropharyngeal lymph node; HP: Hard palate. C. Suppurative omphalitis tracking along umbilical vein (UV) and arteries (UA); Li: Liver; D: Suppurative peritonitis and hepatitis associated with umbilical infection. Li: Liver; E: Head of a stillborn pup. Note bilateral exophthalmos secondary to dystocia; F: Ventral aspect of head and neck of a stillborn pup. Note extensive haemorrhage and oedema of neck and shoulders induced by dystocia. Oe: oesophagus; LF: left fore limb; RF: right fore limb

Organisms isolated in a septicaemic distribution from these cases consisted of *Pasteurella multocida* (2), *E. coli* (2), *Listeria monocytogenes* (2), *Pseudomonas aeruginosa* (1), *Salmonella* Typhimurium (1) and a non-characterised Gram negative non-fermenting bacterium (1). *Salmonella* Bovismorbificans was isolated from 4 tissues of one pup, in co-culture with *Streptococcus phocae* in lung and brain. One pup presented with a septicaemic spread of 4 bacteria (*Arcanobacterium phocae*, *Streptococcus halichoeri*, *Streptococcus phocae* and *Listeria monocytogenes*), typical of that found in the ‘omphalitis’ group. This pup did not present with peritonitis, so did not fulfil the criteria for inclusion in the ‘omphalitis’ category, despite a mixed infection containing *Streptococcus halichoeri* being cultured from the umbilicus.

○ **Dystocia/stillbirth**

Five pups were categorised as stillborn. These animals were recognised by a flaccid carcass with a sticky wet coat, often tinged with meconium and in 2 cases the placenta was located in close proximity to the pup. In the remaining 3 cases the placenta was not found or was too remote from the carcass to be sampled without causing great disturbance to the colony. All five pups had a glistening, pale pink umbilical remnant averaging 9.1cm cm long.

Two of these had atelectatic lungs suggesting they had not taken a first breath and the remaining 3 had expanded lung alveolae suggesting that they had breathed following birth. Four of 5 pups (80.0%) presented with marked bilateral, non-symmetrical exophthalmos (Figure 3-2E), of which two had blood within the anterior chamber (hyphaema) and all 4 of these cases had extensive ecchymotic to suffusive haemorrhage and oedema within the subcutis of the head, neck and shoulder region (Figure 3-2F). One pup (CD039) did not present with any head or neck trauma but had a moderate amount of yellow/brown fluid in the pharynx and larynx, extending into large bronchi, where the fluid was admixed with stable white froth.

Histologically, this pup had evidence of moderate alveolar emphysema, moderate septal and

pleural oedema along with small amounts of meconium within bronchi and alveolae. The gross and histological lesions are supportive of a diagnosis of peri-natal asphyxiation, presumably by allantoic fluid due to lack of rupture of allantoic membranes during parturition. One pup had a serosanguinous abdominal effusion (presumed pressure induced), 3 had stable froth in the large bronchi (suggestive of asphyxiation) and 4/5 (80.0%) had pulmonary interstitial oedema. No lesions were seen in either of the two placenta samples on gross and histological examination. Histologically, 2 pups had evidence of minimal to mild, lymphoplasmacytic to mixed perivascular cuffing within the brain (CD014 and 019) and two pups had structures consistent with nematode profiles within cerebral blood vessels of the anterior cerebral cortex (Figure 3-3) (CD006 and 014). These were characterised by a coelomic cavity, small to medium sized digestive tract and a tripartite oesophagus on cross-section.

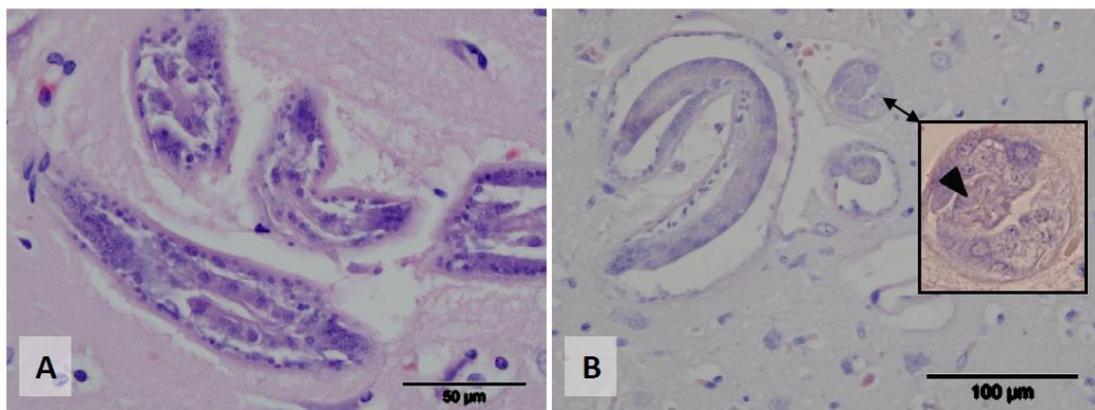


Figure 3-3 A. Brain. Multiple oblique and longitudinal sections of nematode parasite. Stillborn pup. Isle of May. H&E. B. Brain. Transversal and longitudinal sections of nematode parasite. Inset shows the chitin lined tripartite oesophagus (arrowhead). Stillborn pup. Isle of May. H&E.

○ Trauma

Two pups presented with fatal traumatic conditions. The first pup was found at the base of a 5 metre high cliff in very good body condition with localised haemorrhages over the skull and brainstem and a moderate amount of blood in the abdominal cavity (suspected fall). The second pup was found on flat, grassy land with significant peri-ocular haemorrhage and oedema over

the right eye, suffusive haemorrhage over the right temporal muscle and sub-mandibular oedema which had drained to the retropharyngeal lymph nodes. The brain was grossly swollen with mild cerebellar herniation and, on histological examination, evidence of diffuse cerebral oedema. Given the location of this pup, trampling by an adult seal seemed the most likely cause of death. The gastrointestinal tract of both pups showed evidence of recent feeding, and there was no histological evidence of thymic atrophy or any other significant underlying pathology.

○ **Miscellaneous causes of death**

Four pups did not fit accurately into any of the above categories. The first pup was noticeably small at birth and died three days after birth weighing 5.8kg. This pup showed evidence of trauma with haemorrhage and oedema of the ventral neck and larynx in addition to septicaemic spread of the opportunistic pathogen *Aeromonas hydrophila*. The second pup was in excellent body condition at 41.6kg and succumbed to a fibrino-necrotising laryngitis due to penetration of a foreign body (plant material) into the larynx. A combination of *Streptococcus phocae*, *Arcanobacterium phocae* and *E. coli* bacteria were isolated from the lung and larynx.

The third pup weighed 43.8kg and presented with red, serous abdominal effusion; severe pulmonary oedema; widespread panniculitis and fat necrosis associated with possible pancreatic necrosis and gliosis and moderate numbers of Gitter cells within the cerebral cortical grey matter. Bacteriology was unrewarding and the diagnosis of this case remains open.

The fourth pup was in good body condition and presented with marked abdominal and thoracic haemorrhagic effusion, along with marked pleural and pulmonary interstitial oedema.

Haemorrhage was noted around the adventitia of the caudal vena cava and several areas of infarction were recorded in the spleen and brain. Mild vasculitis, neuronal necrosis and gliosis were also present in the brain and a single profile of a helminth parasite was present in the centre of the splenic infarct. The differential diagnosis for this case includes trauma or parasitic migration.

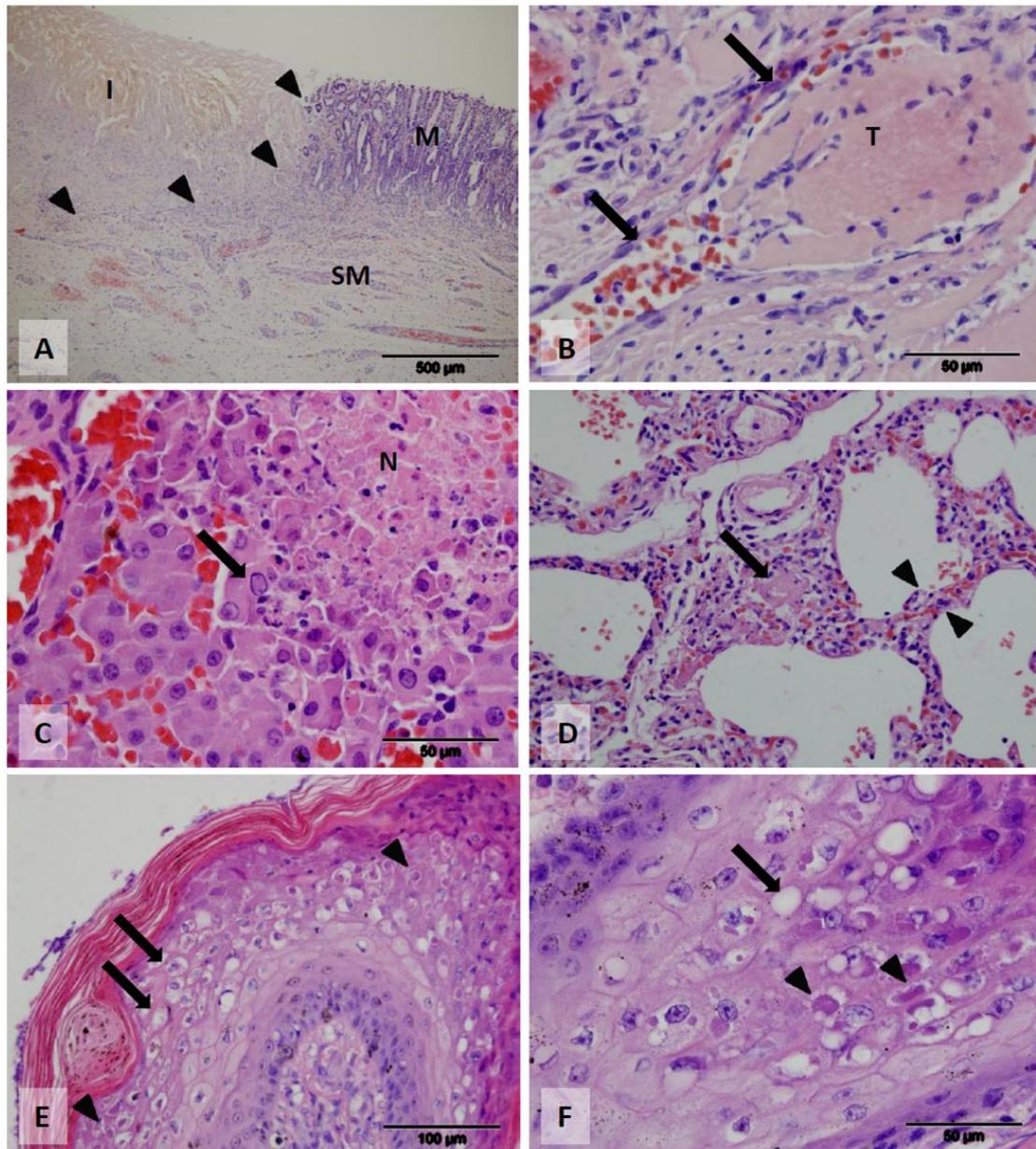


Figure 3-4 Histopathological lesions found in grey seal pups. A: Stomach. Segmental gastric infarction, necrosis and ulceration. M=Normal mucosa; SM= submucosa with moderate perivascular inflammatory infiltrates. I: area of infarction delineated by arrowheads. H&E. B: Stomach. Thrombus (T) within gastric blood vessel (arrow) leading to infarction seen in figure 1. H&E. C: Liver. Hepatic necrosis (N) with putative intranuclear viral inclusion bodies (arrow). H&E. D: Lung. Fibrino-necrotising interstitial pneumonia. Fibrin (arrow); thickened alveolar walls (arrowheads). H&E. E: Skin. Epidermal hyperplasia with ballooning degeneration (arrows) and intracytoplasmic eosinophilic inclusion bodies (arrowheads), typical of poxviral dermatitis. H&E. F: Skin. Ballooning degeneration of keratinocytes (arrow) with characteristic eosinophilic poxviral inclusion bodies (arrowheads). H&E.

Chapter 3 – Neonatal pathology

Table 3-4 Principal gross and histopathological findings in 59 grey seal pups per ultimate cause of death. nb (%): Number of pups affected and percentage of group. ▲: Macroscopical observations; *: Microscopical observations

	Free-living seal pups						Stranded	Total
	Starvation n = 15 nb (%)	Omphalitis n = 13 nb (%)	Septicaemia n = 11 nb (%)	Stillbirth n = 5 nb (%)	Trauma n = 2 nb (%)	Misc. n = 4 nb (%)	n = 9 nb (%)	n = 59 nb (%)
Sex								
Female	4 (26.7%)	9 (69.2%)	2 (18.2%)	2 (40%)	2 (100%)	2 (50%)	3 (33.3%)	24 (40.7%)
Male	11 (73.3%)	4 (30.8%)	9 (81.8%)	3 (60%)	0 (0%)	2 (50%)	6 (66.7%)	35 (59.3%)
Pup stage								
Whitecoat stage 1	4 (26.7%)	2 (15.4%)	0 (0%)	5 (100%)	0 (0%)	1 (25%)	0 (0%)	12 (20.3%)
Whitecoat stage 2	9 (60%)	5 (38.5%)	4 (36.4%)	0 (0%)	1 (50%)	1 (25%)	1 (11.1%)	21 (35.6%)
Whitecoat stage 3	1 (6.7%)	3 (23.1%)	3 (27.3%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	8 (13.6%)
Moult stage 4	1 (6.7%)	3 (23.1%)	3 (27.3%)	0 (0%)	0 (0%)	2 (50%)	4 (44.4%)	13 (22%)
Adult coat stage 5	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	4 (44.4%)	5 (8.5%)
Head and neck								
Mouth ulcers	9 (60%)	3 (23.1%)	6 (54.6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	18 (30.5%)
Thyroid hyperplasia ▲	4 (26.7%)	1 (7.7%)	1 (9.1%)	1 (20%)	0 (0%)	0 (0%)	1 (11.1%)	8 (13.6%)
Thyroid hyperplasia *	3 (20%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)	5 (8.5%)
Tongue ulcers	6 (40%)	3 (23.1%)	4 (36.4%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	14 (23.7%)
Skull – haemorrhage	1 (6.7%)	0 (0%)	1 (9.1%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)	4 (6.8%)
Nasal mites	5 (33.3%)	5 (38.5%)	5 (45.5%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	16 (27.1%)
Number of nasal mites:								
1 to 10	3 (20%)	4 (30.8%)	4 (36.4%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	12 (20.3%)
10 to 50	1 (6.7%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (3.4%)
>50	1 (6.7%)	1 (7.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (3.4%)
Rhinitis/pharyngitis ▲	1 (6.7%)	3 (23.1%)	4 (36.4%)	1 (20%)	1 (50%)	0 (0%)	2 (22.2%)	12 (20.3%)
Eyes								
Hyphaema	0 (0%)	0 (0%)	0 (0%)	2 (40%)	0 (0%)	0 (0%)	0 (0%)	2 (3.4%)
Corneal ulceration	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (33.3%)	3 (5.1%)
Hypopion	0 (0%)	1 (7.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.7%)
Exophthalmos	0 (0%)	1 (7.7%)	0 (0%)	4 (80%)	1 (50%)	0 (0%)	0 (0%)	6 (10.2%)
Keratitis	0 (0%)	1 (7.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (33.3%)	4 (6.8%)
Uveitis	1 (6.7%)	1 (7.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)	3 (5.1%)
Conjunctivitis	5 (33.3%)	5 (38.5%)	2 (18.2%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	13 (22%)
Skin and subcutis								
Congested genital mucosa	4 (26.7%)	0 (0%)	1 (9.1%)	1 (20%)	0 (0%)	2 (50%)	0 (0%)	8 (13.6%)
Congested ano-rectal mucosa	0 (0%)	0 (0%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)	0 (0%)	1 (1.7%)
Diarrhoea	7 (46.7%)	1 (7.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (33.3%)	11 (18.6%)
Congested subcutis	6 (40%)	5 (38.5%)	3 (27.3%)	2 (40%)	0 (0%)	0 (0%)	3 (33.3%)	19 (32.2%)
Icterus	2 (13.3%)	1 (7.7%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	0 (0%)	4 (6.8%)
Haematopoietic system								
Peripheral lymphadenopathy	6 (40%)	3 (23.1%)	8 (72.7%)	1 (20%)	1 (50%)	2 (50%)	4 (44.4%)	25 (42.4%)
Tonsillitis *	11 (73.3%)	6 (54.6%)	8 (72.7%)	0 (0%)	1 (50%)	2 (50%)	6 (66.7%)	34 (59.6%)
Thymic Atrophy (Mild to severe)	13 (92.9%)	8 (61.5%)	8 (80%)	0 (0%)	0 (0%)	0 (0%)	6 (85.7%)	35 (63.6%)
Minimal	1 (7.1%)	2 (15.4%)	1 (10%)	0 (0%)	0 (0%)	2 (50%)	0 (0%)	6 (10.9%)
Mild	4 (28.6%)	1 (7.7%)	1 (10%)	0 (0%)	0 (0%)	0 (0%)	1 (14.3%)	7 (12.7%)
Moderate	9 (64.3%)	7 (53.9%)	7 (70%)	0 (0%)	0 (0%)	0 (0%)	4 (57.1%)	27 (49.1%)
Severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (14.3%)	1 (1.8%)
Splenitis	0 (0%)	5 (38.5%)	3 (27.3%)	0 (0%)	0 (0%)	2 (50%)	1 (11.1%)	11 (18.6%)
Splenic histiocytosis	12 (80%)	9 (69.2%)	9 (81.8%)	1 (20%)	1 (50%)	3 (75%)	2 (22.2%)	37 (62.7%)
Abdomen								
Abdominal effusion	1 (6.7%)	9 (69.2%)	1 (9.1%)	2 (40%)	1 (50%)	2 (50%)	0 (0%)	16 (27.1%)
Type of abdominal effusion								
Haemorrhagic	0 (0%)	1 (7.7%)	0 (0%)	1 (20%)	0 (0%)	2 (50%)	0 (0%)	4 (6.8%)
Suppurative	1 (6.7%)	6 (46.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	7 (11.9%)
Serosanguinous	0 (0%)	1 (7.7%)	0 (0%)	1 (20%)	1 (50%)	0 (0%)	0 (0%)	3 (5.1%)
Umbilicus present	3 (20%)	1 (7.7%)	1 (9.1%)	5 (100%)	1 (50%)	1 (25%)	1 (11.1%)	13 (22%)
Omphalitis	11 (73.3%)	13 (100%)	4 (36.4%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	29 (49.2%)
Severity omphalitis								
Mild	3 (20%)	1 (7.7%)	2 (18.2%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	7 (11.9%)
Moderate	8 (53.3%)	8 (61.5%)	2 (18.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	18 (30.5%)
Severe	0 (0%)	2 (15.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (3.4%)
Fasciitis	0 (0%)	4 (30.8%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)	6 (10.2%)
Peritonitis ▲	3 (20%)	13 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)	17 (28.8%)
Peritonitis/serositis *	3 (20%)	11 (84.6%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	1 (11.1%)	16 (27.1%)

	Free-living seal pups						Stranded	Total n = 59 nb (%)
	Starvation n = 15 nb (%)	Omphalitis n = 13 nb (%)	Septicaemia n = 11 nb (%)	Stillbirth n = 5 nb (%)	Trauma n = 2 nb (%)	Misc. n = 4 nb (%)	n = 9 nb (%)	
Gastrointestinal tract								
Hepatopathy *	9 (60%)	3 (23.1%)	4 (36.4%)	4 (80%)	2 (100%)	1 (25%)	1 (11.1%)	28 (47.5%)
Mesenteric lymphadenopathy *	0 (0%)	2 (15.4%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	0 (0%)	3 (5.1%)
Pancreatic pathology *	0 (0%)	1 (7.7%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (3.4%)
Hepatic necrosis	8 (53.3%)	7 (58.3%)	2 (20%)	0 (0%)	0 (0%)	0 (0%)	4 (44.4%)	21 (36.8%)
Hepatitis	9 (60%)	6 (46.2%)	3 (27.3%)	1 (20%)	1 (50%)	2 (50%)	3 (33.3%)	25 (42.4%)
Multifocal random	8 (53.3%)	4 (30.8%)	3 (27.3%)	0 (0%)	1 (50%)	1 (25%)	3 (33.3%)	20 (33.9%)
Granulomatous	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.7%)
Eosinophilic	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (50%)	1 (25%)	0 (0%)	2 (3.4%)
Gastric ulceration	6 (40%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (33.3%)	9 (15.3%)
Empty stomach	10 (66.7%)	3 (23.1%)	5 (45.5%)	0 (0%)	0 (0%)	2 (50%)	3 (33.3%)	26 (44.1%)
Pica	3 (20%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (6.8%)
Enteritis: mild to severe*	8 (53.3%)	6 (46.2%)	5 (45.5%)	1 (20%)	2 (100%)	1 (25%)	6 (66.7%)	29 (49.2%)
Minimal	3 (20%)	4 (30.8%)	4 (36.4%)	1 (20%)	0 (0%)	2 (50%)	2 (22.2%)	16 (27.1%)
Mild	6 (40%)	6 (46.2%)	5 (45.5%)	1 (20%)	1 (50%)	0 (0%)	5 (55.6%)	24 (40.7%)
Moderate	2 (13.3%)	0 (0%)	0 (0%)	0 (0%)	1 (50%)	1 (25%)	1 (11.1%)	5 (8.5%)
Abnormal colonic mucosa ▲	4 (26.7%)	9 (69.2%)	1 (9.1%)	1 (20%)	0 (0%)	1 (25%)	0 (0%)	16 (27.1%)
Colitis: mild to severe*	7 (46.7%)	4 (30.8%)	4 (36.4%)	0 (0%)	1 (50%)	0 (0%)	2 (22.2%)	17 (28.8%)
Colitis: moderate to severe	3 (20.0%)	2 (15.4%)	3 (27.3%)	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)	8 (13.6%)
Mild	4 (26.7%)	2 (15.4%)	1 (9.1%)	0 (0%)	1 (50%)	0 (0%)	1 (11.1%)	11 (18.6%)
Moderate	3 (20.0%)	2 (15.4%)	3 (27.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (8.5%)
Severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)	1 (1.7%)
Urogenital tract								
Umbilical arteritis	1 (6.7%)	1 (7.7%)	3 (27.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (8.5%)
Adrenal necrosis	2 (13.3%)	1 (7.7%)	3 (27.3%)	0 (0%)	0 (0%)	0 (0%)	2 (22.2%)	8 (13.6%)
Nephritis	1 (6.7%)	1 (7.7%)	2 (18.2%)	0 (0%)	0 (0%)	0 (0%)	2 (22.2%)	6 (10.2%)
Thoracic cavity								
Thoracic effusion	0 (0%)	3 (23.1%)	2 (18.2%)	2 (40%)	0 (0%)	1 (25%)	0 (0%)	8 (13.6%)
Pulmonary congestion ▲	7 (46.7%)	8 (61.5%)	5 (45.5%)	3 (60%)	1 (50%)	2 (50%)	4 (44.4%)	30 (50.8%)
Pulmonary foreign body ▲	0 (0%)	0 (0%)	2 (18.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (3.4%)
Bronchial froth	0 (0%)	2 (15.4%)	3 (27.3%)	2 (40%)	0 (0%)	1 (25%)	2 (22.2%)	10 (16.9%)
Laryngeal pathology ▲	7 (46.7%)	5 (38.5%)	5 (45.5%)	1 (20%)	0 (0%)	3 (75%)	0 (0%)	21 (35.6%)
Trachea: congestion ▲	1 (6.7%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	2 (22.2%)	4 (6.8%)
Trachea froth	2 (13.3%)	3 (23.1%)	3 (27.3%)	3 (60%)	0 (0%)	0 (0%)	1 (11.1%)	12 (20.3%)
Pulmonary oedema:*								
interstitial	1 (6.7%)	5 (38.5%)	10 (90.9%)	4 (80%)	0 (0%)	3 (75%)	3 (33.3%)	26 (44.1%)
alveolar	2 (13.3%)	2 (15.4%)	3 (27.3%)	0 (0%)	0 (0%)	1 (25%)	4 (44.4%)	12 (20.3%)
Pleuritis*	0 (0%)	1 (7.7%)	2 (18.2%)	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)	4 (6.8%)
Interstitial pneumonia*	6 (40%)	8 (61.5%)	8 (72.7%)	0 (0%)	1 (50%)	2 (50%)	4 (44.4%)	29 (49.2%)
Central nervous system								
Brain: congestion ▲	5 (33.3%)	8 (61.5%)	5 (45.5%)	2 (40%)	2 (100%)	1 (25%)	0 (0%)	23 (39%)
Meningitis ▲	1 (6.7%)	0 (0%)	3 (27.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (6.8%)
Brain - ventriculitis ▲	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.7%)
Brain - oedema ▲	2 (13.3%)	0 (0%)	0 (0%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	3 (5.1%)
Meningitis *	3 (20%)	3 (23.1%)	10 (90.9%)	0 (0%)	0 (0%)	0 (0%)	3 (33.3%)	19 (32.2%)
Encephalitis *	3 (20%)	0 (0%)	9 (81.8%)	0 (0%)	0 (0%)	0 (0%)	2 (22.2%)	14 (23.7%)
Granulomatous	0 (0%)	0 (0%)	3 (27.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (5.1%)
Suppurative	3 (20%)	0 (0%)	5 (45.5%)	0 (0%)	0 (0%)	0 (0%)	2 (22.2%)	10 (16.9%)
Pyogranulomatous	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.7%)
Chronic-active	1 (6.7%)	0 (0%)	3 (27.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (6.8%)
Non suppurative	0 (0%)	1 (7.7%)	3 (27.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (6.8%)
Gliosis	3 (20%)	2 (15.4%)	7 (63.6%)	0 (0%)	0 (0%)	4 (100%)	2 (22.2%)	18 (30.5%)
Gitter cells	1 (6.7%)	2 (15.4%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	0 (0%)	4 (6.8%)
Perivascular cuffing	3 (20%)	2 (15.4%)	6 (54.6%)	2 (40%)	0 (0%)	2 (50%)	4 (44.4%)	19 (32.2%)
Locomotor system								
Arthritis ▲	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	1 (25%)	2 (22.2%)	4 (6.8%)
Spinal abscessation	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.7%)
Cardiovascular system								
Pericarditis*	0 (0%)	3 (23.1%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)	0 (0%)	4 (6.8%)
Haemorrhage endocardium or epicardium	0 (0%)	1 (7.7%)	1 (9.1%)	0 (0%)	0 (0%)	1 (25%)	0 (0%)	3 (5.1%)
Myocarditis (minimal or mild)*	6 (40%)	7 (53.9%)	6 (54.6%)	0 (0%)	0 (0%)	1 (25%)	2 (22.2%)	22 (37.3%)
Vasculitis	9 (60%)	5 (38.5%)	5 (45.5%)	0 (0%)	1 (50%)	1 (25%)	6 (66.7%)	27 (45.8%)
Thrombosis	8 (53.3%)	7 (53.9%)	3 (27.3%)	0 (0%)	0 (0%)	1 (25%)	4 (44.4%)	23 (39%)

○ **Temporal distribution of mortality**

The distribution of the ultimate causes of neonatal mortality in the grey seal pups examined on the Isle of May is presented in Figure 3-5 and Figure 3-6.

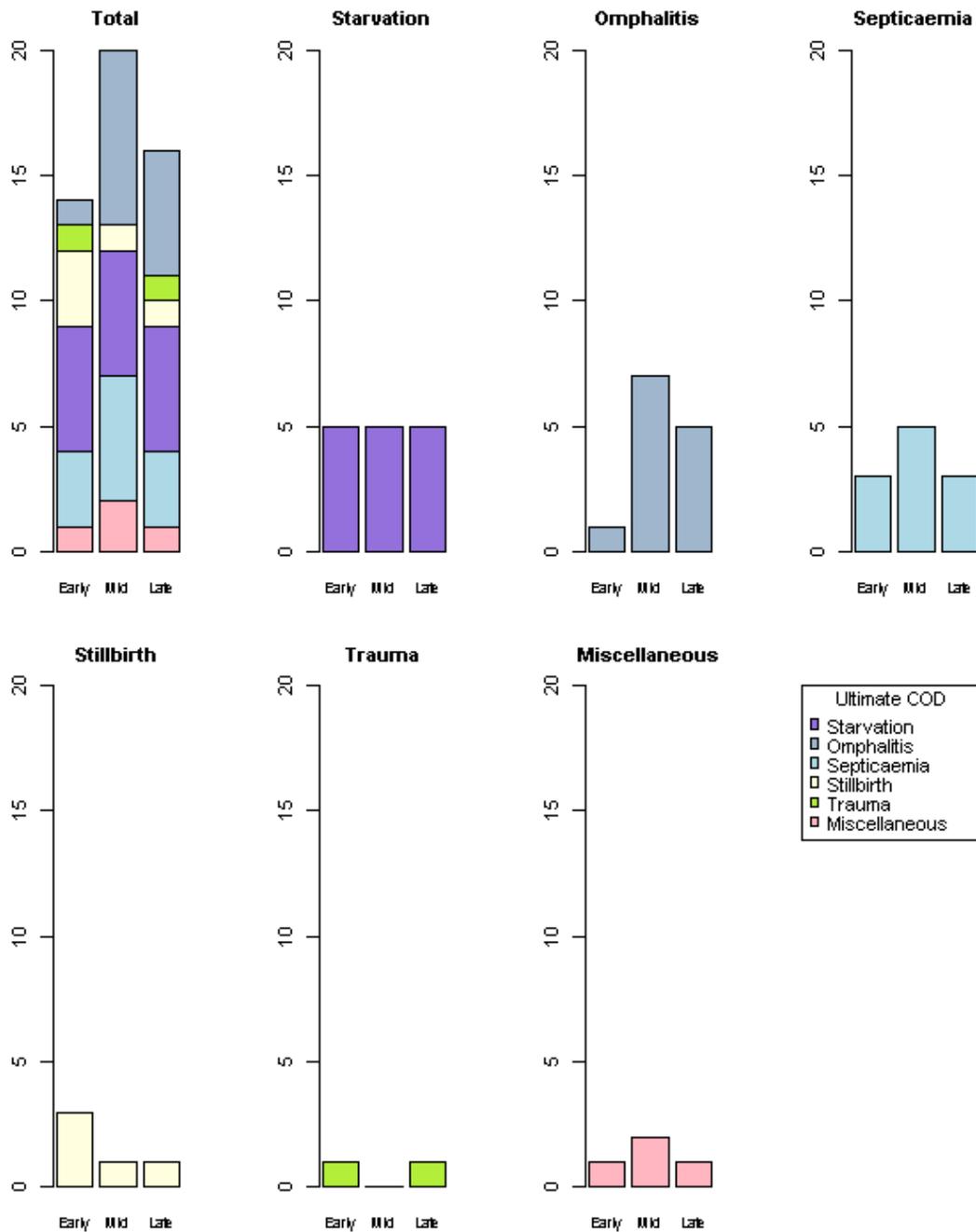


Figure 3-5 Distribution of the primary causes of neonatal mortality in dead grey seal pups examined on the Isle of May during the 2011 pupping season per sampling time (early, mid and late pupping season).

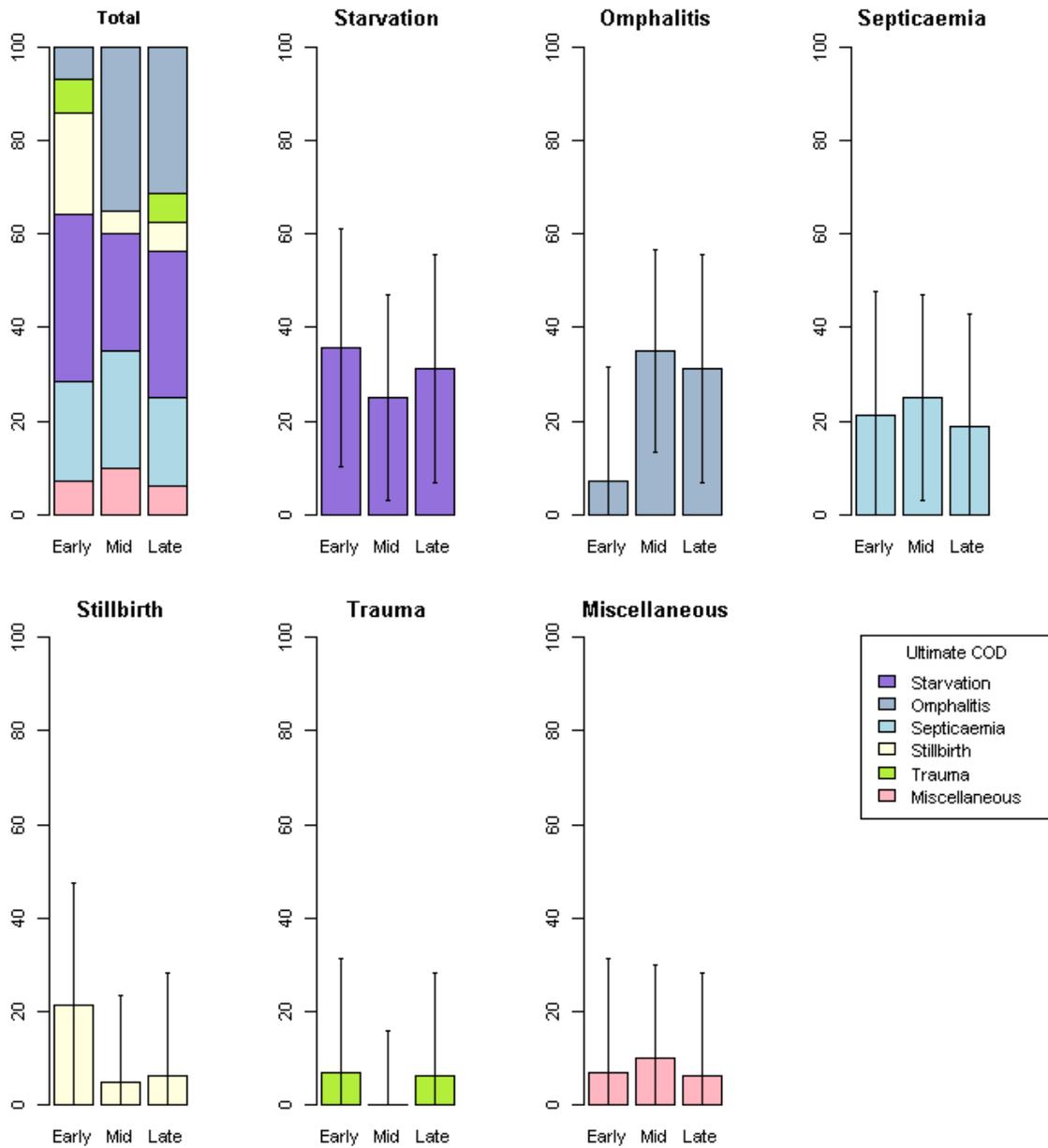


Figure 3-6 Distribution of the primary causes of neonatal mortality in dead grey seal pups examined on the Isle of May during the 2011 pupping season expressed as a percentage of overall mortality per sampling time (early, mid and late pupping season). Bars represent 95% confidence interval.

When examining the distribution of ultimate cause of death per substrate type (grass; boulder beach; rocky pools and the mixed environment of Kirkhaven), the grass environment showed a statistically significantly lower proportion of pups dying of omphalitis-peritonitis complex than on the tidal boulder beach ($p=0.026$, FET). The proportion of pups dying of starvation was

however higher on the grass environment (45.0%) and the tidal boulder beach (30.0%) than on the two other substrates (15.8%) but this difference was not statistically significant.

Contributing factors:

Several (13/50, 26.0%) dead pups from the Isle of May were found to have more than one disease process resulting in death, the contribution of each process was examined when it was considered a significant finding in any specific animal. Infection was a significant factor in the death of 35 (70.0%) pups; starvation contributed to the demise of 16 (32.0%) pups and trauma to 6 (12.0%). The involvement of each of these three processes within the overall cause of death is shown below (Figure 3-7). Starvation and trauma remained relatively constant throughout the pupping season, whereas infection showed a steady increased with time. The difference between the prevalence of infection in early to mid pupping season approached statistical significance (p=0.07).

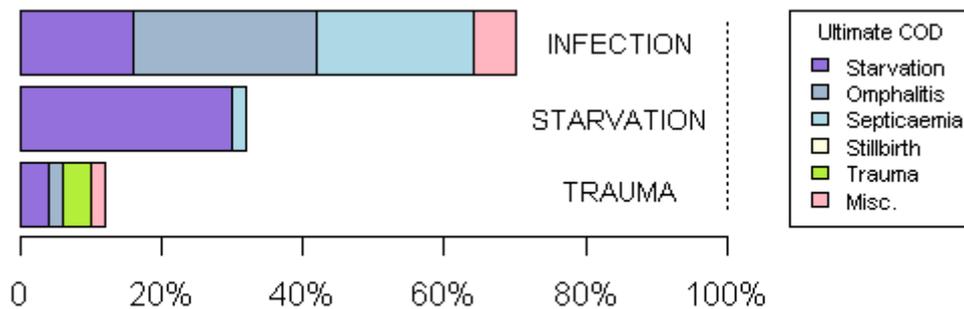


Figure 3-7 Proportion of free-ranging grey seal pups presenting with infection, starvation or trauma at post-mortem examination on the Isle of May. Colour represents the ultimate cause of death attributed to each pup.

Sex

The ratio of male to female pups examined at necropsy was 1.38 in favour of males, whereas a random sample of 90 live grey seal pups carried out in parallel to this study (see Chapter 2)

demonstrated a roughly equal sex ratio of 44 males to 45 females on the colony². This difference between sex ratio in live and dead pups on the Isle of May was, however, not statistically significant ($p=0.38$, FET). When examining the sex distribution per cause of death, being male increased the odds of succumbing to starvation by a factor of 6.19 (95% CI: 1.2, 31.97, $p=0.03$, glm) and of dying of septicaemia by 10.13 (95% CI: 1.47, 69.93, $p=0.019$, glm) when compared to those succumbing to omphalitis.

3.3.2 Stranded grey seal pups - Scottish SPCA National Wildlife Rescue Centre - Ultimate cause of death

Signalment, morphometric data, ultimate cause of death and secondary findings for each of the 9 seal pups presented for post-mortem examination are presented in Table 3-2. Starvation was the most common primary cause of death with 4/9 cases (44.4%); 2 pups succumbed to septicaemia and the remaining 3 were categorised as miscellaneous. Miscellaneous causes of death included two seal pups with bilateral ulcerative keratitis of unknown aetiology, likely due to phocid herpesvirus 1 (see Chapter 6), and a single case of seal poxvirus dermatitis. All three cases resulted in euthanasia.

The poxviral lesions were recognised by multifocal to coalescing, raised, dark pink to red nodular lesions on the haired skin of the palmar aspect of both fore and hind limbs Figure 3-8B. A large area of excoriation was also noted on the ventral aspect of the chin which was associated with extensive cellulitis and oedema of the ventral neck. Severe lymphoid atrophy, mild vacuolar hepatopathy with bile stasis and brain lesions suggestive of hepatic encephalopathy were also present in this animal. Histologically, skin lesions consisted of severe segmental

² The sex of one pup was not recorded

epidermal hyperplasia with extensive ballooning degeneration and brightly eosinophilic intracytoplasmic inclusion bodies typical of seal poxvirus (Figure 3-4E and F).

The sex ratio within dead pups submitted from the rehabilitation centre followed a similar trend to that seen on the Isle of May with an over-representation of male pups (6 males to 3 females).



Figure 3-8 Gross pathology in stranded grey seal pups. A. Suppurative arthritis; shoulder joint associated with *Pseudomonas aeruginosa* infection. Sc: scapula. Stranded grey seal pup. Scottish SPCA National Wildlife Rescue Centre. B. Multifocal to coalescing, proliferative poxviral dermal lesions (arrowheads). Fore flipper of stranded grey seal pup. Scottish SPCA National Wildlife Rescue Centre.

3.3.3 Secondary cause of death and incidental findings

- **Secondary causes of death**

Several lesions of note which, although not considered the ultimate cause of death, may have played a contributing role in the demise of the free-ranging and stranded grey seal pups in this study are detailed below.

Skin puncture wounds/bites were recorded in 50.0% (25/50) of grey seal pups dying on the Isle of May colony and 44.4% (4/9) stranded pups. They were most frequently located around the fore and hind flippers, with less frequent puncture wounds around the head and neck or dorsal aspect of the pelvis.

Eyes were frequently surrounded by a sticky dark brown to black liquid, with plant debris, mud and fur agglomerated in this area with histological evidence of conjunctivitis in 22.0% (13/59) cases. There was no evidence of corneal ulceration in any of the pups on the Isle of May (0/50) but 33.3% (3/9) stranded pups had grossly evident corneal ulceration.

Nasal mites were found in 32.0% pups (16/50) on the Isle of May in all cause of death groups with the exception of the stillborn pups. Pups presenting with in excess of 10 nasal mites within the nasal cavities/pharynx were found in the starvation, omphalitis and septicaemia groups. Although the presence of nasal mites was significantly increased in animals with moderate to severe thymic atrophy ($p=0.013$, FET; or OR=5.73, 95% CI: 1.49, 22.01, $p=0.011$ glm), the severity of nasal mite infection (scored 0 to 4) did not correlate with the presence of moderate to severe thymic atrophy nor with the degree of thymic atrophy. No nasal mites were found in any of the stranded grey seal pups.

Arthritis was present in 4 animals. The first case was associated with bite wounds to the caudal flippers and the second case consisted of a spinal abscess associated with septicaemic spread of concurrent *Salmonella Bovismorbificans* and *Streptococcus phocae* infection. A stranded pup had severe suppurative arthritis, osteomyelitis and cellulitis of the left shoulder joint associated with *Pseudomonas aeruginosa* septicaemia Figure 3-4A. A second stranded pup, which succumbed to starvation, had a suppurative arthritis within the tibio-tarsal joint, most likely secondary to a bite wound.

Tongue ulcers were present in 23.7% pups (14/59), were generally bilaterally symmetrical and located at the lateral edges of the tongue, adjacent to the canine teeth. The presence of mouth ulcers correlated with the presence of tongue ulcers ($p=0.02$, FET).

Mouth ulcers were noted in 30.5% pups (18/59) of pups and were frequently located immediately adjacent to the erupting canine teeth, or at the labial commissure. When ulcers were present, the mouth had a foetid odour. Although not statistically different, the mean mass of pups with mouth ulcers was 14.8kg, whereas those without had a mean mass of 18.2kg.

On internal examination, the most commonly recorded lesion was that of umbilical infection, present in 27 (54.0%) of pups, although this was considered to be the ultimate cause of death in only 13 pups (26.0%). When not associated with fulminant peritonitis and hepatitis, the vessels were occluded by a pale tan, more viscous to caseous pus and, occasionally, small foci of inflammation and fibrin deposition were noted on the opposing mesentery. In corroboration with previous studies, no significant omentalisations were present (Baker et al. 1980). No omphalitis was noted in any of the 9 stranded grey seal pups.

Jaundice was present in 4 animals, in 3 cases this was associated with multifocal hepatic necrosis. The pup without hepatic necrosis was significantly undersize (possibly premature) and had evidence of bile stasis indicative of intrahepatic or post-hepatic jaundice.

Histological evidence of meningitis was present in 19 pups (starvation, septicaemia, omphalitis and stranded seal groups) with a very high prevalence in the septicaemia group (10/11, 90.9%). Gliosis was present in all pups within the miscellaneous group, despite a lack of meningitis or encephalitis. Meningitis ranged from non-suppurative to pyogranulomatous in affected pups and an effort will be made in the bacteriology section to correlate the type of cellular infiltrate to the bacteria isolated from each case.

Small numbers of helminth parasite profiles were present in the brains of 2 stillborn pups (Figure 3-3A and 1-3B) and the spleen of a third. These profiles were located within the lumina

of medium sized blood vessels in the cerebral cortex and within the centre of an infarct in the spleen. No inflammation was associated with these structures.

Drowning or asphyxiation was the proximate cause of death in two cases of non-specific septicaemia on the Isle of May, and in both cases initially misled the initial diagnosis until histology and bacteriology were completed. The first pup was found in a stagnant fresh water pool with evidence of aspiration pneumonia (silt and plant material in large bronchi) and the second was found with its head firmly lodged in a rabbit hole; the lungs were overinflated and very pale pink with moderate laryngeal oedema. Septicaemia due to *Pseudomonas aeruginosa* and *E. coli*, respectively, were the ultimate cause of death of these two pups.

Multifocal, random, hepatic necrosis and adrenal necrosis, typically described with phocid herpesvirus 1 (Figure 3-4C), were both present with a respective prevalence of 36.8% (21/58) and 13.6% (8/59). This prevalence and association with the presence of phocid herpesvirus 1 is discussed further in Chapter 6.

- **Incidental findings/ other lesions**

Numerous variably sized, eosinophilic intracytoplasmic droplets were noted in moderate to large numbers of epithelial cells within sections of haired skin, tongue, buccal and genital mucosa sections (Figure 3-9B). These structures were more frequent in areas of inflammation, hyperplasia or dysplasia. These “pseudo-inclusions” have been noted by several experienced marine mammal pathologists and are thought to consist of aggregates of cytoskeletal proteins, reflecting a subtle change in metabolism of the epithelial cell (L. Lowenstein, personal communication). It is unlikely that these structures represent viral inclusion bodies as they are present in practically all animals examined and are not overtly associated with inflammation.

Particular lesions of note included mild to moderate atrophic enteritis (3) within the starvation group and suppurative tonsillitis (59.6%, 34/57) which did not correlate with the presence of tongue ulcers or mouth ulcers.

Small, raised oesophageal lesions were noted in one pup (CD047) (Figure 3-9A), consisting histologically of focal hyperplasia, dysplasia, mild superficial ulceration and chronic-active inflammation. The aetiology of this lesion was not clear, but the presence of concurrent laryngeal ulceration, atrophic dermatitis and markedly enlarged thyroid glands, favoured a diagnosis of hypothyroidism or nutritional deficiency (such as hypovitaminosis A).

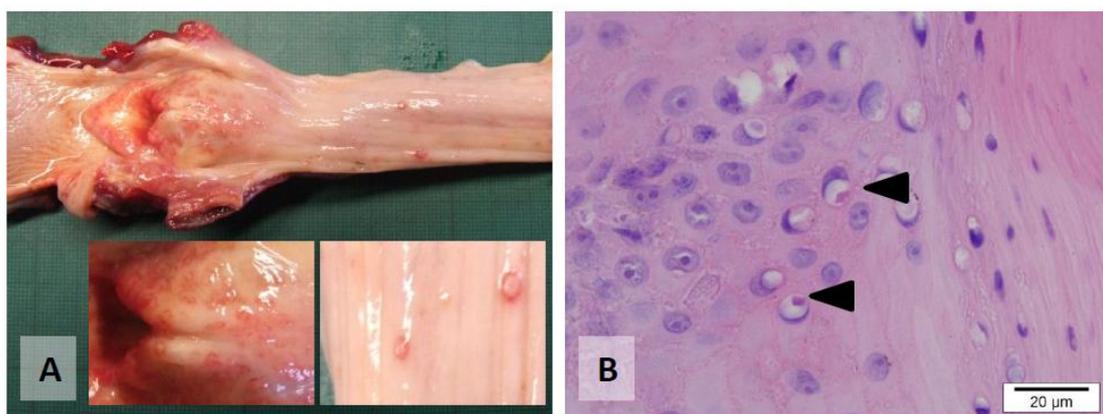


Figure 3-9 Gross and histopathological lesions found in grey seal pups. A: Laryngeal ulceration and multifocal, small, raised oesophageal lesions in a pup that died of starvation; B: Amphophilic to eosinophilic intracytoplasmic “pseudo-inclusions” in buccal epithelium of a free-ranging grey seal pup with buccal ulceration.

3.3.4 Systemic bacteriology

A wide range of bacterial species were isolated from the grey seal pup tissues and are presented in Table 3-5.

- **Isle of May colony**

Bacterial species isolated from dead pups examined on the Isle of May colony were diverse but some bacterial species were more frequently associated with particular causes of death (as described previously; see 3.3.1).

- ***Mycoplasma* spp.**

Mycoplasma spp. were sporadically isolated from laryngeal or lung samples. In all cases these were co-infections with other bacterial and viral agents (see Chapter 6). In one case (CD030), *Mycoplasma phocicerebrale* was isolated in conjunction with septicaemic spread of *Pasteurella multocida* and *Streptococcus canis* plus individual isolations of *Streptococcus agalactiae* (lung) and *A. phocae* (lung). In another case, *Mycoplasma phocicerebrale* was found in conjunction with *Actinomyces* spp., *Salmonella* Bovismorbificans and *Streptococcus dysgalactiae* (CD048). An unidentified, probably novel, species of *Mycoplasma* was isolated from the larynx of seal CD027 in conjunction with *Pasteurella multocida* isolated from lung samples and a septicaemic spread of *Streptococcus agalactiae*, *Streptococcus halichoeri* and *Streptococcus phocae*.

- ***Streptococcus* spp.**

S. phocae was the most frequently isolated species of *Streptococcus*, isolated from 30.0% (15/50) pups and frequently associated with omphalitis (13/15) and peritonitis (8/15) (see Table 3-3). Numerous other species of *Streptococcus* were isolated from seal pup tissues: *Streptococcus agalactiae* (3 pups), *Streptococcus canis* (3), *Streptococcus dysgalactiae* ssp. *equisimilis* (1), *Streptococcus equi* ssp. *zooepidemicus* (2), *Streptococcus gallolyticus* ssp. *pasteurianus* (1), *Streptococcus halichoeri* (3) and an unidentified *Streptococcus* sp. (1). Streptococcal species were consistently found in co-infections, suggesting that they were opportunistic bacterial species. In addition, 90.9% (20/22) of *Streptococcus* infected pups had some degree of umbilical infection.

○ *Listeria monocytogenes*

Listeria monocytogenes was isolated from a total of 13 pups within the starvation, omphalitis and septicaemia groups. The detection of *L. monocytogenes* was significantly more common in pups with mild to severe thymic atrophy ($p=0.049$, FET; OR=5.19, 95% CI: 1,26, 94, $p=0.029$) than in pups with no or minimal thymic atrophy. Although not statistically significant, *L. monocytogenes* was more frequently isolated from pups with umbilical infection (OR=1.22, $p=0.764$) than from those without umbilical infection; this bacterium was also more frequently isolated from pups with histological evidence of peritonitis (OR=2.67, $p=0.147$) or hepatic necrosis (OR=2.27, $p=0.228$) than from pups not presenting these lesions.

L. monocytogenes was localised within spleen (1/1), lung (2/2) and within foci of hepatic necrosis in the liver (2/3) sections selected for immunohistochemistry (Figure 3-10). No labelling was present within brain sections (0/3).

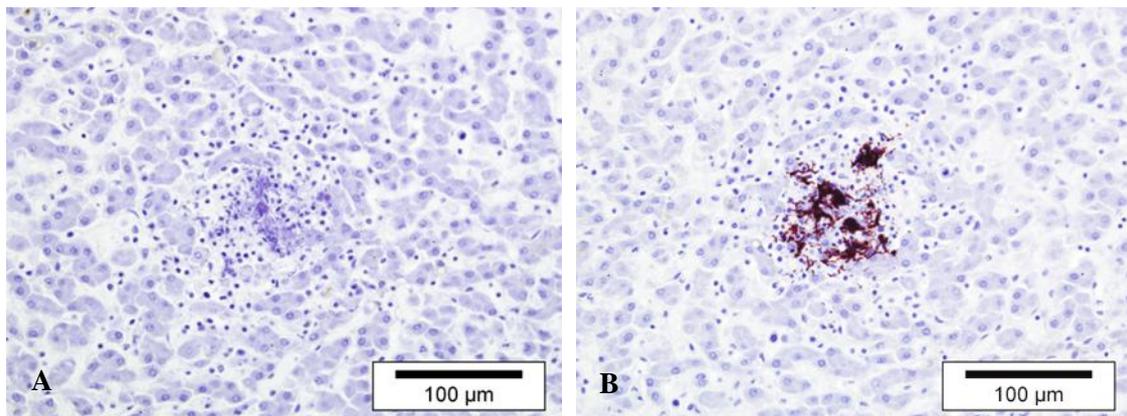


Figure 3-10 *Listeria monocytogenes* IHC. (A) Negative control (omitting primary antibody); (B) Intense labelling of *L. monocytogenes* within foci of hepatic necrosis and chronic-active inflammation. x200. IHC

○ *Pasteurella multocida*

This pathogen was the apparent cause of death in two pups, associated with suppurative pleuropneumonia, and likely a significant contributing factor in the death of a third pup, which succumbed to omphalitis. Immunohistochemistry demonstrated weak labelling within the pleura and alveolae of one pup (CD030). The antibody used was developed against *Pasteurella*

multocida serotype A3 and the weak labelling may reflect infection with a different serotype of *P. multocida*.

- **Scottish SPCA National Wildlife Rescue Centre**

Of the 9 pups submitted from the rehabilitation centre, isolation of bacteria from tissues was sparse, relatively non-specific and often mixed (Table 3-5). Exceptions to this were the isolation of *Pseudomonas aeruginosa* in 3 pups (R004, R007 and R008). A key histological finding in these three pups was vasculitis. Pup R004 presented with neuronal necrosis, thrombosis within the pulmonary vasculature, peritonitis and renal tubular degeneration and necrosis; pup R007 presented with suppurative meningo-encephalitis associated with vasculitis and pup R008 presented with suppurative to pyogranulomatous meningoencephalitis with intravascular bacterial colonies, vasculitis and thrombosis. Pups R004 and R007 presented with significant skin lesions around the head and shoulder regions, including a large, shoulder abscess with significant osteolysis of the scapula (Figure 3-8A), which were likely the point of entry of this bacterium.

Arcanobacterium phocae was isolated from the brain of a single pup (R006) although autolysis was too advanced to fully interpret the significance of this finding.

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Table 3-5 Bacteria isolated from 59 stranded and free-ranging grey seal pups in different categories of ultimate cause of death. Numbers represent number of pups per group in which each bacterium was isolated; numbers in brackets represent number of samples of each tissue type in which each bacterium was isolated.

Bacteria	Total	Free-ranging pups					Stranded pups	
	Total (n=59)	Miscellaneous (n=4)	Septicaemia (n=11)	Starvation (n=15)	Stillborn (n=5)	Trauma (n=2)	Omphalitis (n=13)	Stranded (n=9)
<i>Acinetobacter</i> sp.	1 Brain (1), Lung (1)							1 Brain (1), Lung (1)
<i>Actinomyces</i> sp.	1 Liver (1), Lung (1), Spleen (1)			1 Liver (1), Lung (1), Spleen (1)				
<i>Aeromonas sobria</i>	1 Brain (1), Liver (1)							1 Brain (1), Liver (1)
<i>Aeromonas hydrophila</i>	2 Brain (1), Liver (1), Lung (2), Spleen (1)	1 Brain (1), Liver (1), Lung (1), Spleen (1)		1 Lung (1)				
<i>Arcanobacterium phocae</i>	13 Brain (4), Larynx (1), Liver (5), Lung (4), Spleen (9), Umbilicus (3)	1 Larynx (1), Lung(1)	2 Brain (1), Lung (1), Spleen (1)	2 Brain (1), Spleen (1), Umbilicus (1)			7 Liver (5), Lung (2), Spleen (7), Umbilicus (2)	1 Brain (1)
Coagulase negative <i>Staphylococcus</i> sp.	1 Brain (1)							1 Brain (1)
Putative <i>Corynebacterium</i> sp.	1 Spleen (1)							1 Spleen (1)
<i>E. coli</i>	17 Brain (4), Liver (7), Lung (14), Spleen (5), Umbilicus (2)		2 Brain (1), Liver (1), Lung (2), Spleen (2)	7 Brain (1), Liver (3), Lung (6), Spleen (2), Umbilicus (2)		1 Brain (1), Lung (1)	6 Brain (1), Liver (3), Lung (4), Spleen (1)	1 Lung (1)

Bacteria	Total	Free-ranging pups						Stranded pups
	Total (n=59)	Miscellaneous (n=4)	Septicaemia (n=11)	Starvation (n=15)	Stillborn (n=5)	Trauma (n=2)	Omphalitis (n=13)	Stranded (n=9)
<i>Enterobacter</i> sp.	1 Liver (1)			1 Liver (1)				
<i>Enterococcus faecalis</i>	3 Liver (1), Lung (2)			1 Lung (1)			1 Lung (1)	1 Lung (1)
<i>Erysipelothrix rhusiopathiae</i>	1 Brain (1), Liver (1), Lung (1), Spleen (1)			1 Brain (1), Liver (1), Lung (1), Spleen (1)				
<i>Escherichia fergusonii</i>	1 Brain (1), Liver (1), Lung (1), Spleen (1)			1 Brain (1), Liver (1), Lung (1), Spleen (1)				
Gram negative non fermenter	10 Brain (4), Liver (1), Lung (4), Spleen (3)		1 Liver (1), Lung (1), Spleen (1)	3 Brain (1), Lung (2)			2 Brain (1), Lung (1)	4 Brain (2), Spleen (2)
Gram positive rods	2 Brain (2), Liver (1), Spleen (1)			1 Brain (1), Liver (1), Spleen (1)		1 Brain (1)		
Gram variable cocco-bacilli	1 Spleen (1)	1 Spleen (1)						
Group D <i>Streptococcus</i> spp.	1 Lung (1)							1 Lung (1)
Haemolytic <i>E. coli</i>	1 Lung (1)						1 Lung (1)	

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Bacteria	Total	Free-ranging pups					Stranded pups	
	Total (n=59)	Miscellaneous (n=4)	Septicaemia (n=11)	Starvation (n=15)	Stillborn (n=5)	Trauma (n=2)	Omphalitis (n=13)	Stranded (n=9)
<i>Hafnia alvei</i>	1 Lung (1)			1 Lung (1)				
<i>Listeria monocytogenes</i>	13 Brain (1), Liver (9), Lung (4), Spleen (9)		4 Liver (4), Lung (3), Spleen (2)	4 Liver (4), Spleen (2)			5 Brain (1), Liver (1), Lung (1), Spleen (5)	
Mixed	34 Abscess (1), Brain (22), Larynx (2), Liver (1), Lung (7), Placenta (2), Spleen (7), Umbilicus (2)	3 Brain (3), Lung (1)	8 Brain (5), Liver (1), Lung (2), Umbilicus (1)	8 Abscess (1), Brain (5), Larynx (1), Liver (1), Lung (1), Spleen (3), Umbilicus (1)	3 Brain (2), Liver (2), Lung (2), Placenta (2), Spleen (1)		8 Brain (5), Larynx (1), Lung (4), Spleen (2)	4 Brain (2), Lung (3), Spleen (1)
<i>Mycoplasma phocicerebrale</i>	2 Larynx (1), Lung (1)		1 Lung (1)	1 Larynx (1)				
<i>Mycoplasma sp.</i>	2 Larynx (2)			1 Larynx (1)			1 Larynx (1)	
NG at 14 days	32 Brain (12), Liver (21), Lung (11), Spleen (16)	3 Liver (1), Lung (1), Spleen (2)	3 Brain (1), Liver (1), Lung (1), Spleen (1)	7 Brain (4), Liver (5), Lung (3), Spleen (4)	4 Brain (3), Liver (3), Lung (3), Spleen (4)	2 Liver (2), Lung (1), Spleen (2)	6 Brain (4), Liver (2), Spleen (1)	7 Liver (7), Lung (2), Spleen (2)
Non-haemolytic <i>E. coli</i>	2 Larynx (1), Liver (2), Lung (1)	1 Larynx (1), Liver (1), Lung (1)					1 Liver (1)	
<i>Pasteurella multocida</i>	3 Brain (2), Liver (2), Lung (3), Spleen (2)		2 Brain (2), Liver (2), Lung (2), Spleen (2)				1 Lung (1)	

Bacteria	Total	Free-ranging pups					Stranded pups	
	Total (n=59)	Miscellaneous (n=4)	Septicaemia (n=11)	Starvation (n=15)	Stillborn (n=5)	Trauma (n=2)	Omphalitis (n=13)	Stranded (n=9)
<i>Proteus</i> sp.	12 Abscess (1), Brain (5), Larynx (2), Lung (7), Spleen (1), Umbilicus (1)			7 Abscess (1), Brain (3), Larynx (1), Lung (3), Spleen (1), Umbilicus (1)			3 Brain (1), Larynx(1), Lung (2)	2 Brain (1), Lung (2)
<i>Pseudomonas aeruginosa</i>	5 Abscess (1), Brain (3), Liver (2), Spleen (3)		1 Brain (1), Liver (1), Spleen (1)	1 Liver (1), Spleen (1)				3 Abscess (1), Brain (2), Spleen (1)
<i>Pseudomonas</i> sp.	1 Liver (1)	1 Liver (1)						
<i>Salmonella Bovismorbificans</i>	3 Abscess (1), Brain (2), Liver (3), Lung (1), Spleen (3)		1 Brain (1), Liver (1), Lung (1), Spleen (1)	1 Liver (1), Spleen (1)			1 Brain (1), Liver (1), Spleen (1)	
<i>Salmonella Typhimurium</i>	1 Liver (1)		1 Liver (1)					
<i>Staphylococcus delphini/ pseudintermedius</i>	1 Brain (1)					1 Brain (1)		
<i>Streptococcus agalactiae</i>	3 Liver (1), Lung (2), Spleen (2)		1 Lung (1)	1 Spleen (1)			1 Liver (1), Lung (1), Spleen (1)	
<i>Streptococcus canis</i>	3 Brain (1), Liver (2), Lung (1), Spleen (3), Umbilicus (2)		1 Brain (1), Liver (1), Spleen (1)				2 Liver (1), Lung (1), Spleen (2), Umbilicus (2)	

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Bacteria	Total	Free-ranging pups						Stranded pups
	Total (n=59)	Miscellaneous (n=4)	Septicaemia (n=11)	Starvation (n=15)	Stillborn (n=5)	Trauma (n=2)	Omphalitis (n=13)	Stranded (n=9)
<i>Streptococcus dysgalactiae</i> ssp. <i>equisimilis</i>	1			1				
	Liver (1), Spleen (1), Umbilicus (1)			Liver (1), Spleen (1), Umbilicus (1)				
<i>Streptococcus equi</i> ssp. <i>zooepidemicus</i>	2						2	
	Liver (1), Lung (1), Spleen (1), Umbilicus (1)						Liver (1), Lung (1), Spleen (1), Umbilicus (1)	
<i>Streptococcus gallolyticus</i> ssp. <i>pasteurianus</i>	1						1	
	Lung (1)						Lung (1)	
<i>Streptococcus halichoeri</i>	3		1				2	
	Brain (1), Liver (3), Lung (2), Spleen (2), Umbilicus (1)		Brain (1), Liver (1), Lung (1), Spleen (1), Umbilicus (1)				Liver (2), Lung (1), Spleen (1)	
<i>Streptococcus phocae</i>	15	1	3	3			8	
	Brain (3), Larynx (1), Liver (8), Lung (7), Spleen (6), Umbilicus (5)	Larynx (1), Lung (1)	Brain (2), Liver (2), Lung (2), Spleen (1)	Lung (1), Umbilicus (2)			Brain (1), Liver (6), Lung (3), Spleen (5), Umbilicus (3)	
<i>Streptococcus</i> sp.	3		1			1		1
	Lung (3)		Lung (1)			Lung (1)		Lung (1)
Unidentified coliform	1		1					
	Liver (1), Spleen (1)		Liver (1), Spleen (1)					

3.4 Discussion

3.4.1 Pathology of free-ranging grey seal pups

Despite significant changes in the Isle of May grey seal population, the main causes of death when compared to those reported almost 30 years previously (Baker & Baker 1988) do not appear to have changed dramatically. Much improved sampling techniques and previously unavailable technology such as ultra-low temperature freezer facilities, allowed for optimal sample collection, storage, down-stream processing and pathogen identification and consequently a much more detailed investigation of infectious disease. This was acknowledged as an inevitable limitation of many previous studies (Baker et al. 1980; Baker 1984). This study provides an objective overview of causes of neonatal mortality and pathology on the Isle of May colony during the 6 week 2011 pupping season. Systematic examination of all available freshly dead animals and rigorous bacterial screening enabled a complete baseline study of the mortality and associated bacterial flora on a grey seal colony.

Starvation, omphalitis and septicaemia, the main causes of death within neonatal pups on the Isle of May colony have been previously recognised as major causes of mortality in grey seal pups irrespective of the colony studied (Baker 1984; Baker 1988; Baker et al. 1998; Baker & Baker 1988). Their respective prevalence is variable within the published literature depending on the colony studied and age of animals examined, but starvation, causing 30% of deaths in the present study, accounted for 40 to 58% of pup deaths on the Isle of May colony in 1986 (Baker & Baker 1988). Bacterial infections were a contributing factor in the death of 70% pups in the present study with 26% pups succumbing to the omphalitis-peritonitis complex and 22% to bacterial septicaemias other than omphalitis, comparable to the 14-50% prevalence of omphalitis-peritonitis and 8-21% prevalence of septicaemia estimated in 1986 (Baker & Baker 1988). Although direct comparison of findings at these two time-points is difficult, as categories

of ultimate cause of death are different between years and the 1986 study often attributed more than one cause of death to each pup, fewer pups appear to have succumbed to starvation in the present study than in earlier studies.

Starvation as a major cause of death is a common finding in other pinniped breeding colonies such as Antarctic fur seals (Baker & Doidge 1984), New Zealand sea lions (Castinel *et al.* 2007) and South American fur seals (Seguel *et al.* 2008). This is largely a consequence of maternal separation or failure of the mother-pup bond. Sites with higher densities of breeding females or sites where displacement occurs due to the incoming tide, such as that seen on the boulder beach of Pilgrim's Haven, could be expected to have a higher proportion of pups dying of starvation than other sites. The present study found that this hypothesis held true for the tidal boulder beach site (3 of 10; 30%). The high proportion of pups dying of starvation on grassy areas (9 of 20; 45%) is harder to explain but may have been artificially high due to exclusion of abandoned pups from the main body of the colony rather than a particular characteristic of the grassy substrate.

The high occurrence of omphalophlebitis in grey seal pups seems to be a relatively specific feature of grey seals, with several studies showing a high incidence of this condition on breeding colonies (Baker *et al.* 1980; Baker 1984; Baker & Baker 1988). Omphalitis certainly occurs in other Phocid species such as harbour seals but is rarely referred to as a major cause of mortality in this species, manifesting predominantly as umbilical abscesses without peritonitis (Johnson *et al.* 1998; Lockwood *et al.* 2006; Steiger *et al.* 1989). In Otariids, Castinel *et al.* (2007) highlighted the small number of omphalitis cases in an extensive study of neonatal mortality in the New Zealand Sea lion and Spraker and Lander (2010) found omphalophlebitis in only 1% of Northern fur seal pups.

The higher proportion of pups dying of omphalitis on the tidal boulder beach when compared to other sites could be a consequence of the increased pup density at high tide or alternatively due to exposure to sea-water. In contrast, grassy areas with the lowest occurrence of pups dying of omphalitis, tended to be drier and less soiled than other substrate types. The increased occurrence of omphalitis over the course of the pupping season, most likely reflects increased contamination of the pupping ground over time. In previous studies, increased occurrence of omphalitis has been found in pups born on sandy beaches and in pups born later in the season (Baker & Baker 1988). Although pups from the sandy beach were not accessible in 2011, these findings support Baker's theory that omphalitis is associated with dirtier, more abrasive pupping grounds (Baker 1984; Baker & Baker 1988).

The contribution of both starvation and trauma to the death of grey seal pups on this colony seems to be a relatively constant phenomenon throughout the course of the pupping season. In contrast, the relative proportion of animals succumbing to infection increased in mid and late pupping season compared to early season. This is probably due to the pupping grounds becoming increasingly soiled by faeces, lanugo and the carcasses of dead pups, all contributing to increased environmental bacterial load and therefore more efficient spread within the seal colony.

Although not statistically significant, the apparent over-representation of dead male pups (29/50) is noteworthy. Such a trend has previously been described in grey seal pup colonies (Baker 1984) and, perhaps of more relevance for the population, the first year survival rate of grey seal pups has been shown to be significantly higher in females compared to males (Hall et al. 2001). Higher neonatal mortality in male animals is not uncommon in young domestic species such as pigs as well as humans (Baxter et al. 2012; Naeye et al. 1971). Several physiological and behavioural explanations have been proposed for this difference including the immunosuppressive effect of androgens (Olsen & Kovacs 2001).

One other point of consideration would be the energetic “cost” of investing in a male pup. Arnbohm *et al.* (1994) reported that Southern elephant seals “must weigh more than 300 kg if they are to breed at all, and more than 380 kg if they are to give birth to a male pup” (Arnbohm *et al.* 1994). This implies that the energy reserves necessary to take a male pup to term may not be sufficient if a female is below a certain size. In grey seals, male pups have been shown to be heavier at weaning than females (Hall *et al.* 2001). It is not implausible that small, inexperienced females may be more likely to abandon a male pup more often than a female pup in a bid to limit depletion of maternal reserves and protect their future reproductive potential. What percentage of the overall contribution to survival rate is decided in the neonatal period is worthy of further investigation.

In contrast, the over-representation of female pups succumbing to omphalitis may be an incidental finding, or reflect an immunological or anatomical difference in susceptibility compared to male pups. Early infection of the urachus could escalate to septicaemia quicker in male pups, given the aforementioned lower immunity, thus leading to death before peritonitis occurs; alternatively, the conformation of the urachus could be different between sexes such that bacterial ingress and establishment may be easier in female pups.

The discovery of nematode parasites in the tissues of 3 seal pups, and notably in 2 stillborn pups is proof of trans-placental transmission of these parasites. The identity of the parasite is not yet known but nucleic acids have been extracted from these sections for further identification. *Parafilaroides* sp., which has been implicated in the transmission of *Brucella pinnipedialis* in phocid seals (Garner *et al.* 1997), would a likely candidate. The prospect of transplacental transmission opens a whole new arm in the ecology of this parasite and possibly of *Brucella* spp..

3.4.2 Pathology of stranded grey seal pups

The small number of stranded grey seal pups examined at post-mortem examination (n=9) makes it difficult to compare different causes of death with those seen in free-ranging grey seal pups. Starvation was a prominent feature in these stranded grey seal pups but chronic processes, not seen on the Isle of May colony, such as corneal ulceration and poxvirus lesions, were noted. It is not unreasonable to assume that the process of live stranding may have selected for chronic conditions. The lack of omphalitis was noteworthy, although this condition was recorded in 36.4% (4/11) pups submitted from the same rehabilitation centre in 2010 (JB, unpublished data). Given that the age of the pups examined on the Isle of May and in the rehabilitation centre are comparable, the small sample size is likely to be the key limiting factor in this group.

3.4.3 Systemic bacteriology of grey seal pups

A key strength of this study was the opportunity to perform systematic and high quality bacteriology from field samples without the substantial sampling delay which hindered most historical studies (Baker et al. 1980; Baker et al. 1998). Long term refrigeration of swabs or storage at ambient temperature is likely to have resulted in a failure of isolation of many organisms due to die off or overgrowth of other bacterial species. Consequently, it is difficult to fully compare data from the present study and historical samples.

Arcanobacterium phocae, *E. coli*, *Streptococcus phocae* and *Listeria monocytogenes* form a dominant cluster in grey seal pups succumbing to umbilical infection-septicaemia. These bacteria are likely to represent aggressive opportunists, benefitting from any breach in the integrity of the abdominal wall to cause aggressive disease in their hosts. This group of pathogens, in particular *S. phocae* and *A. phocae*, warrant further investigation given their frequent isolation from marine mammal tissues (Hueffer et al. 2011; Johnson et al. 2003; Skaar et al. 1994).

A. phocae was frequently isolated (23.3%, 12/59 pups) although was seldom present in isolation (1 case). This bacterium is thought to be an aggressive, opportunistic pathogen, reminiscent of *A. haemolyticum* in humans, and likely to be part of the normal skin or oral flora of marine mammals (Johnson et al. 2003). Generally associated with superficial pyogenic infections, spread can be rapid, leading to deep seated and systemic infections (Johnson et al. 2003). *A. phocae* is thought to be an important cause of clinical disease in marine mammals and the pathology associated with this organism would benefit from further study (Johnson et al. 2003). The significant association between *A. pyogenes* and *Streptococcus phocae* in the present study may be indicative of symbiosis or simply reflect a shared portal of entry of these two bacterial species.

Comparing the prevalence of *A. phocae* with that described in historical studies of grey seal pathology is largely hindered by the fact that this species was not characterised and described until 1997 following phylogenetic analysis of the genus *Actinomyces* (Ramos et al. 1997). Given the high prevalence of *A. phocae* in the present study, it would not be implausible to expect this bacterium to have been historically present on the seal colony. As *A. phocae* grows readily on standard culture media both in air and CO₂ enriched atmospheres (Ramos et al. 1997) it is unlikely that this bacteria were not isolated if present. It is tempting to speculate that the *Corynebacterium pyogenes* described in early field studies could be the *A. phocae* isolated in the present study. *Corynebacterium pyogenes*, has been reclassified several times, initially as *Actinomyces pyogenes* (Collins & Jones 1982), later as *Arcanobacterium pyogenes* (Ramos et al. 1997) and is currently known as *Trueperella pyogenes*. *T. pyogenes* is a pathogen of significant veterinary importance, in particular in cattle and pigs (Hirsch & Biberstein 2004) and displays many similarities to *A. phocae* but is not a known pathogen of British marine mammals (N. Davison and G. Foster, pers. comm.). Culture and identification techniques used in the early

seal studies are unfortunately not sufficiently discriminatory to retrospectively confirm this hypothesis.

The isolation of *Listeria monocytogenes* is a novel finding in grey seals, and, to the author's knowledge, the first report of this bacterium in any marine mammal. This bacterium was found in 30% (15/50) of grey seal pups on the Isle of May and was not present in any of the pups from the rehabilitation centre. *L. monocytogenes* was, however, isolated from a grey seal pup at the same rehabilitation centre during the 2010 pupping season (G. Foster and J.B., unpublished data). *L. monocytogenes* is a facultative anaerobic bacterium of significance in human and veterinary health, leading to febrile gastroenteritis, septicaemia, meningoencephalitis and late term spontaneous abortion. In monogastric animals and neonates, septicaemia with multifocal to diffuse hepatic and splenic necrosis is the most frequent presentation (Walker 2004).

Immunosuppression predisposes to development of clinical disease in many cases. Ingestion of contaminated food or soil is the most common source of exposure of animals and humans. The role of Phocid Herpesvirus 1 in the presence of *L. monocytogenes* will be further discussed in Chapter 6.

The isolation of *Listeria monocytogenes* in such a high proportion of grey seal pups raises concerns of zoonotic disease risk to humans in contact with carcasses of grey seal pups. The prevalence and significance of this pathogen in adult grey seal populations is completely unknown but it is tempting to speculate that it may play a role in older, immunosuppressed individuals or act as an abortifacient agent as has been described in other species. Further typing of the *L. monocytogenes* isolates using pulsed field gel electrophoresis (PFGE), multilocus variable number of tandem repeat analysis (MLVA) and multi-locus sequence type (MLST) schemes may help elucidate the origin and potential zoonotic risk of this organism in seals.

The large variety of *Streptococcus* species isolated in the present work correlates with earlier studies (Anderson et al. 1979; Baker 1984; Baker & Baker 1988). However, given the rudimentary classification of Streptococci at that time, strains were described using phenotypical characteristics and classed in Lancefield groups which hinders comparison between these time points (Baker 1984; Baker & Baker 1988). *Streptococcus phocae*, Lancefield Group C and F is likely to correspond to the *Streptococcus* strain S2 described by Baker as the second most common Streptococcus species in three breeding colonies in 1984 (Baker 1984). *Streptococcus phocae* was recognised as a separate species in 1994 (Skaar et al. 1994) and has since been found in a wide range of marine mammals, generally associated with suppurative inflammation (Hueffer et al. 2011). *S. canis*, a beta-haemolytic *Streptococcus* species of Lancefield groups C and G is most commonly associated with domestic dogs. This pathogen has been found in harbour seal pups on Smith Island, Washington, USA, in two pups presenting with omphalophlebitis and omphalitis-peritonitis (Leahy 2010). No Streptococci described in earlier studies of grey seal pup mortality matches the phenotypical characteristic of *S. canis*. *S. equi* ssp. *zooepidemicus*, a beta-haemolytic *Streptococcus* species of Lancefield group C, was also not described in earlier studies. This pathogen has been recorded in dead-stranded grey seals with pyothorax, septicaemia and from oral ulcers in England and Wales (Baker et al. 1998) and in harbour seals during the 2002 PDV epidemic during which it was thought to contribute to the pathology seen in these animals (Akineden et al. 2007). *S. equi* ssp. *zooepidemicus* is known to be pathogenic to domestic dogs, leading to acute fatal pneumonia and is occasionally zoonotic (Priestnall et al. 2010). No *Streptococci* of Lancefield group C, likely to correspond to *Streptococcus agalactiae* in the present study were described in early surveys.

The isolation of an *Actinomyces* sp. (species unknown) in one animal in septicaemic distribution is an unusual finding. The significance of the presence of this bacterium is unknown as this pup also presented emaciation and a septicaemic distribution of *Salmonella Bovismorbificans*.

Furthermore, *Streptococcus agalactiae* was isolated from the spleen and *Mycoplasma phocicerebrale* from the larynx of this animal. On balance, this suggests invasion of a highly debilitated host and that the *Actinomyces* sp. isolated in this case represents an opportunistic pathogen. Hoyles *et al.* (2001) described a novel species of *Actinomyces*, *A. marimammalium* in a hooded seal, grey seal and a harbour porpoise (Hoyles *et al.* 2001).

It is interesting to note that despite using specific culture media, no *Bordetella* spp. were isolated from any pups. *Bordetella* spp. was a frequent finding in grey and harbour seals following the 1998 and 2002 phocine distemper outbreaks, so widespread that it was almost considered a commensal, the pathogenicity of which was not understood (Munro *et al.* 1992). The lack of *Staphylococcus aureus* is also noteworthy as is the lack of *Brucella* spp., given the increasing reports of this bacterium in pinnipeds (Foster *et al.* 1996; Foster *et al.* 2002; Prenger-Berninghoff *et al.* 2008).

The sparse and generally non-specific nature of the bacteria isolated from pups at the rehabilitation centre may well be explained by previous antibiotic therapy in these animals (7/9 pups).

3.4.4 Limitations of the study

This study is tempo-spatially limited as it represents a snapshot of neonatal mortality in Autumn 2011 on the Isle of May colony. Any extrapolation from this study to all grey seal breeding colonies and from year to year within the Isle of May should be carried out with caution.

With a larger data-set, principal component analysis could be considered in order to extract the most significant lesions and pathogens associated with each cause of death group. This was not

envisaged in the current study as the number of animals (observations) was deemed too small for meaningful analysis but may be a consideration with a larger dataset.

The complexity of disease progression was oversimplified by determining a single ultimate cause of death for each pup. This resulted in the loss of distinction between several sub-groups of pups. Indeed many pups which initially were suffering from starvation/malnutrition succumbed to infection, most likely due to protein/calorie induced immunosuppression, whilst other pups may have presented with starvation secondary to septicaemia due to being too ill to feed despite the opportunity being present.

In addition, the method of selection of dead grey seal pups for post-mortem examination may have induced a small bias towards pups dying of non-traumatic conditions. Any pups dying due to drowning, trauma by falling off cliffs or storm damage are unlikely to have presented in suitably fresh condition to have been included in this study, given the difficulty of locating and retrieving these carcasses in a suitable timeframe.

3.4.5 Conclusions and suggestions for further studies

This study details the causes of neonatal mortality on a well established grey seal breeding colony, providing a crucial baseline of what represents “normal” mortality in this population. Distinguishing background or “normal” mortality from unusual mortality events is essential in marine mammal management and protection. Indeed, deviations from this baseline could be an indicator of exposure to novel pathogens or toxins, decreased nutritional status or anthropogenic ecological change.

The causes of pup mortality on the Isle of May colony do not appear to have significantly changed over the last 30 years. Starvation, omphalitis, septicaemia and stillbirth were common

causes of death on this site. Pathogens such as *Arcanobacterium phocae* and *Streptococcus phocae* were common, and frequently associated with omphalitis. *Listeria monocytogenes* was isolated for the first time in any marine mammal species and was associated with omphalitis and septicaemia. The presence of nematode parasites within brain tissues of stillborn pups is the first proof of transplacental transmission of such parasites in this species and adds an extra dimension to the ecology of nematode infection in grey seals. Causes of death in stranded grey seal pups presented to a wildlife rescue centre were different from those seen on their natal colony with an over-representation of chronic conditions in this group.

As this study focused only on one site, over one pupping season, prospective studies would be warranted to monitor trends in mortality, benefitting from the long term population monitoring carried out at this site. Ideally, this sampling process would be repeated over a series of successive pupping seasons, or at regularly defined intervals, on the Isle of May colony to see if the causes of mortality fluctuate between years. Comparison between sites would also be warranted, given the difference in grey seal population dynamics between sites (Sea Mammal Research Unit 2012).

Causes of mortality in weaned grey seal pups and mortality in pups up to one year of age remain poorly understood. Given that first year pup survival is as low as 19.3% for male pups (Hall et al. 2001), knowing what these pups are subjected to once they leave the colony would further help understand what may drive grey seal populations. Logistical constraints, predominantly weather conditions, limited the present study to approximately 6 weeks but further studies are required to determine the causes of mortality over a longer period, in multiple age-classes, in particular post-weaning and during the first year of life after departure to sea.

Chapter 4 Investigation of *Salmonella enterica* in neonatal and juvenile Scottish grey seals

4.1 Introduction

The genus *Salmonella* is a member of the family Enterobacteriaceae and is composed of two species, *S. bongori* and *S. enterica*. *S. enterica* is further subdivided into six subspecies (I to VI), the most common of which, in mammals and birds, is *Salmonella enterica* subspecies I (also referred to as *Salmonella enterica* subspecies *enterica*) (Brenner et al. 2000). Each subspecies is further divided into serotypes, which are identified on the basis of the serologic identification of O (somatic) and H (flagellar) antigens, possessed by most *Salmonellae*, forming the basis of the Kauffman-White-Le Minor classification scheme (Grimont & Weill 2007; Guibourdenche et al. 2010). Within *S. enterica* subsp. I the most common O-antigen serogroups are A, B, C1, C2, D and E (Brenner et al. 2000). By convention, the majority of serotypes are represented by names which are capitalised and depicted in roman print (Brenner et al. 2000). Thus *Salmonella enterica* subspecies *enterica* serotype Typhimurium is referred to as *S. Typhimurium*. This convention will be used here from this point on. Since *Salmonella* exhibit phase variation between motile and non-motile phenotypes (or specific and non-specific phases), different "H" antigens may be expressed depending on the phase examined, as presented in Table 4-1 (Grimont & Weill 2007).

Serotype names are designated by antigenic formulae which are composed of: (i) subspecies designation (subspecies I through VI), (ii) O (somatic) antigens followed by a colon, (iii) H (flagellar) antigens (phase 1) followed by a colon, and (iv) H antigens (phase 2, if present) (for example *Salmonella Bovismorbificans*: *Salmonella* serotype I 6,8,20: r,[i]: 1,5 (Brenner et al. 2000).

Table 4-1 - *Salmonella* nomenclature based on the Kauffman-White classification scheme, based on Grimont and Weill (Grimont & Weill 2007)

O-group	Serotype	O antigens	Phase 1 (motile) H antigens	Phase 2 (non-motile) H antigens	Antigenic formula
A	<i>S. Paratyphi A</i>	1,2,12	a	[1,5]	1,2,12: a: [1,5]
B	<i>S. Typhimurium</i>	1,4,[5],12	i	1,2	1,4,[5],12: i: 1,2
	<i>S. Haifa</i>	1,4,[5],12	z ₁₀	1,2	1,4,[5],12: z ₁₀ : 1,2
	<i>S. Brandenburg</i>	4,5,12	l,v	e,n,z ₁₅	4,5,12: l,v: e,n,z ₁₅
C ₁	<i>S. Montevideo</i>	6,7,14	g,m,[p], s	[1,2,7]	6,7,14: g,m,[p],s: [1,2,7]
C ₂	<i>S. Bovismorbificans</i>	6,8,20	r,[i]	1,5	6,8,20: r,[i]: 1,5
	<i>S. Newport</i>	6,8,20	e,h	1,2	6,8,20: e,h: 1,2
D	<i>S. Typhi</i>	9,12,[Vi]	d	no phase 2 antigen	9,12[Vi]: d: –
	<i>S. Dublin</i>	1,9,12 [Vi]	g,p	no phase 2 antigen	1,9,12[Vi]: g,p: –

Salmonellae are Gram negative, non-spore forming rods measuring 2-3µm in length. They are facultative anaerobes, oxidase negative and grow readily on selective media such as MacConkey agar or Brilliant Green agar. They are non-lactose fermenting and their growth on brilliant green agar plates is recognised by bright pink to red colonies. Isolation of *Salmonella* spp. from faecal samples commonly requires use of an enrichment medium. Faeces can be incubated for 12-18 hours in selenite F broth, which suppresses the growth of any organisms other than *Salmonella* prior to culture and identification using a selective agar medium (Hirsch 2004a; Hirsch 2004b).

Infection with *Salmonella* spp. is a major global human and animal health concern causing more than 90 million human cases of disease worldwide annually (Majowicz et al. 2010). It is the second most commonly reported cause of bacterial infectious intestinal disease in Scotland after *Campylobacter* spp. and most cases can be attributed to contaminated food products (Browning et al. 2012). While generally leading to transient gastro-intestinal symptoms such as diarrhoea,

vomiting, fever, anorexia and malaise, it can produce potentially fatal invasive infections (Coburn et al. 2007).

Salmonella spp. are widely found in domestic and wild animal species, leading to three different disease presentations: a systemic septicaemia/typhoid due to serotypes such as *S. Choleraesuis*; a gastro-intestinal colonisation varying from asymptomatic infection to acute or chronic enteritis due to serotypes such as *S. Enteritidis*; and finally, more rarely, reproductive forms leading to abortion (Brown et al. 2007; Uzzau et al. 2000). Gastrointestinal forms can progress to more severe disease with systemic involvement resembling typhoid in the immunosuppressed (such as very young or very old animals or patients) (Uzzau et al. 2000).

Salmonella spp. bacteria have been isolated from several pinniped species including grey seals, harbour seals (*Phoca vitulina*), Stellar sea lions (*Eumetopias jubatus*), New Zealand sea lions (*Phocarctos hookeri*), Antarctic fur seals (*Arctocephalus gazella*), Northern Elephant seals (*Mirounga angustirostris*) and California Sea lions (*Zalophus californianus*) (Baker et al. 1995; Carrasco et al. 2011; Fenwick et al. 2004; Palmgren et al. 2000; Stoddard et al. 2008a; Stoddard et al. 2008b), in locations remote from human habitation and with increased frequency in recent years. It is unknown if this apparent increase in infection is the result of human activity or exposure between marine mammals and other animals, marine or terrestrial, or a reflection of increased sampling effort. The detection of multidrug resistant strains of *Salmonella* spp. from several marine mammal species (Foster et al. 1998; Johnson et al. 1998; Stoddard et al. 2005) has raised concerns about microbial environmental pollution. Determining the type and origin of these multidrug-resistant strains may establish the source of *Salmonella* isolates and what part, if any, anthropogenic activity plays in the spread of this bacterium.

Within the UK pinniped populations, *Salmonella* spp. isolates have been isolated from faeces and faecal swabs of both apparently healthy and clinically ill grey and harbour seals. A 1995

study found a prevalence of between 0 - 2.5% in free-ranging harbour seals in the Wash and the Isle of Skye and in stranded pups on arrival at a Norfolk seal rehabilitation centre (Baker et al. 1995). *Salmonella* Typhimurium phage type 49 was isolated from harbour seals in the Wash, whereas *S. Bovismorbificans* and *S. Newport* were isolated from harbour seals in the Norfolk rehabilitation centre. A prevalence of 11.8% was found in grey seals arriving at the same rehabilitation centre in Norfolk but no *Salmonella* spp. isolates were recovered from grey seals on the Isle of Skye (Baker, 1995). *S. Bovismorbificans* was the only *Salmonella* serotype isolated from grey seals on arrival at the rehabilitation centre. In the same study, two clinically ill seals at a Scottish rehabilitation centre died of haemorrhagic gastroenteritis from which *S. Bovismorbificans* was isolated (Baker, 1995). Prior to this, *Salmonella Bovismorbificans* had been described in 5 dead grey seal pups (Anderson et al. 1979; Baker 1980), three of which had acute focal hepatitis and two of which presented with septicaemia.

Baker *et al.* (1995) hypothesised that the higher prevalence of *Salmonella* spp. in grey seals was a consequence of their dirtier, denser pupping grounds which may favour survival and transfer of *Salmonella* spp. between individuals. Links between human strains of *Salmonella* Typhimurium phage type 49 and cattle strains of *S. Bovismorbificans* pointed towards a possible land-sea transfer of these bacteria. In 1998, *Salmonella* Typhimurium phage type 104, which is a virulent and drug-resistant pathogen of numerous livestock species and humans (Threlfall 2000), was isolated from a juvenile grey seal from the Moray Firth, again raising speculation of potential disease transmission between livestock and marine mammals along the coastline (Foster et al. 1998). However, more structured prospective studies are required to further investigate a potential livestock-marine mammal transmission of *Salmonella* spp.. The advent of advanced genetic fingerprinting techniques such as pulsed field gel electrophoresis (PFGE) and other methodologies has enabled better understanding of the putative origin and spread of many food borne pathogens (Swaminathan et al. 2001). Analysis of the diversity of

Salmonella spp. strains isolated from grey seal populations and how they compare to isolates in the Scottish *Salmonella*, *Shigella* and *Clostridium difficile* reference laboratory (SSSCDRL) database means that the source and distribution of the marine *Salmonella* can now be investigated.

Although DNA sequencing of highly variable genes will likely become the method of choice for molecular epidemiologists in the future, currently the “gold standard” technique used in this discipline is pulsed-field gel electrophoresis (PFGE) (Murase et al. 1995). PFGE is utilised worldwide as a key tool in the surveillance of foodborne diseases. By rigorously standardising protocols, molecular weight markers, and methods for the exchange of standardised gel data, large international networks such as Pulsenet International (Swaminathan et al. 2001) are able to collate and compare isolates from numerous bacterial species worldwide and respond rapidly to outbreaks irrespective of geographical borders (Ribot et al, 2006).

PFGE is essentially the comparison of large genomic DNA fragments after digestion with one or more restriction enzymes. The bacterial genome being circular, means that several variably sized DNA fragments are produced and subsequently size separated by electrophoresis through an agarose gel using an alternating voltage gradient, where the electric field changes direction through 120°, to improve the resolution of these larger molecules (Buckingham 2007). Each bacterial genome thus digested and separated gives a particular banding pattern depending on the location of the sites at which the restriction enzymes act on the DNA. Assuming two strains are clonal, the sites at which the restriction enzymes act on the DNA will be identical, leading to an identical PFGE banding pattern; therefore if two isolates show the same banding pattern they are considered to represent the same strain (Buckingham 2007). In cases where PFGE is not sufficiently discriminatory, new subtyping methods such as multiple-locus variable number tandem repeat analysis (MLVA) have been developed based on genome analysis (Ramisse et al. 2004). These have the added advantage of there being minimal experimental variation in DNA

sequence results, allowing direct inter-laboratory comparisons and the potential for greater accuracy in determining the likelihood that two or more strains are related (Lindstedt et al. 2004). Virtually all pro- and eukaryotic genomes contain short repeat sequences that are dispersed throughout the genome. In each repeat sequence locus the repeat copy number can vary between different strains, hence these sequences are often referred to as “variable-number tandem repeats” or VNTRs. In MLVA a multiplex PCR amplifies each locus of interest using fluorescently labelled forward primers. The labelled PCR products can be detected and their sizes determined using high resolution capillary electrophoresis in an automatic sequencer. At each locus, the size of the fragment enables the number of repeat units at each locus to be deduced (Lindstedt et al. 2003; Lindstedt et al. 2004). The copy number at each locus is called the allele number and the combination of the allele numbers for each locus gives a “barcode” for each strain, which can easily be compared to reference databases. MLVA is currently widely used for protocols to classify *Salmonella enterica* serotype Typhimurium using 7 loci (CDC 2013; ECDC 2007).

The primary goal of this part of the study was to determine the prevalence and types of *Salmonella* spp. in live and dead, free-ranging grey seal pups and yearlings and in stranded live and dead pups. To help elucidate the origin of any seal isolates and their relationship with known terrestrial and human isolates, typing was performed with four different but complementary typing methods: pulsed-field gel electrophoresis (PFGE), Multilocus Variable Number of Tandem Repeat Analysis (MLVA), phage typing and serotyping. In addition, to investigate whether antimicrobial resistance was present in *Salmonella* isolated from grey seals, antimicrobial sensitivity patterns were also investigated.

Before field sampling, an assessment of enrichment and cryostorage techniques for *Salmonella* spp. was carried out to allow for restricted field laboratory facilities.

4.2 Freezing trial

To assess the best method of sampling for *Salmonella* species in faeces, given the constraints of inevitably long delays between sampling and processing of samples, an enrichment trial with a -80°C freezing stage was carried out.

4.2.1 Methods

- **Quantification of inoculum**

Cultures of *Salmonella enterica* serovar Bovismorbificans, kindly donated by Geoff Foster, SAC VCS, Inverness, were resuscitated from Microbank beads (Prob-Lab diagnostics, Wirral, UK) onto Luria-Bertani (LB) broth agar plates (Becton Dickinson, Oxford, UK) and incubated for 24h at 37°C. A single colony was placed into each of 4 glass vials containing 10ml of LB broth and incubated for 24h at 37°C. The optical density of the broth solution was assessed using LB broth as a blanking solution at a wavelength of 600nm using a WPA CO 6000 spectrophotometer (Biopharm Ltd, Cambridge). An optical density of a neat bacterial suspension measured 0.81 (OD of a 10⁻¹ solution measured 0.9).

Serial dilutions of *Salmonella* broth were prepared in duplicate, using phosphate buffered saline (PBS) as diluant. Dilutions were prepared in duplicate from 10⁻¹ to 10⁻¹⁰ neat broth. LB agar plates were prepared for the Miles, Misra and Irwin method of assessing bacterial concentrations (Miles et al. 1938). The Miles, Misra and Irwin Method (or surface viable count) is a technique used in microbiology to determine the number of colony forming units in a bacterial suspension or homogenate. Plates were divided into 9 to 12 areas (triplicates of 3-4 dilutions per plate) (Figure 4-1). Ten microlitres of the appropriate dilution was dropped onto the surface of the agar and the drop allowed to spread naturally. The plates were left upright on the bench to dry before inversion and incubation at 37°C for 24 hours.

Each sector was observed for growth. High concentrations gave a confluent growth over the area of the drop, or a large number of small/merged colonies. Colonies were counted in the sector where the highest number of full-size discrete colonies were seen (usually sectors containing between 2-20 colonies). The following equation was used to calculate the number of colony forming units (CFU) per ml from the original sample:

$$CFU \text{ per ml} = \text{Average number of colonies for a dilution} \times 100 \times \text{dilution factor.}$$

Duplicate samples were prepared for incubation at room temperature and at 37°C. Thus 12 plates of LB agar were prepared as demonstrated in Figure 4-1. Dilutions 10^{-1} to 10^{-4} gave confluent growth in all cases; dilution 10^{-5} showed full size discrete colonies with numbers in excess of 40; dilution 10^{-6} showed full size, discrete colonies (Figure 4-2).

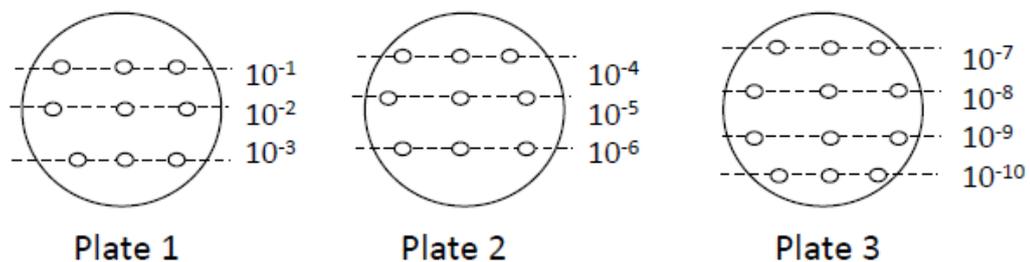


Figure 4-1 Preparation of LB agar plates for Miles, Misra and Irwin method of assessing bacterial concentrations. Each circle represents the location of a 10µl drop of bacterial suspension at the dilution stated next to the figure. Each of the three plates was prepared in duplicate for each sample with one plate incubated at 37°C for 24h and the other incubated at room temperature for 24h.

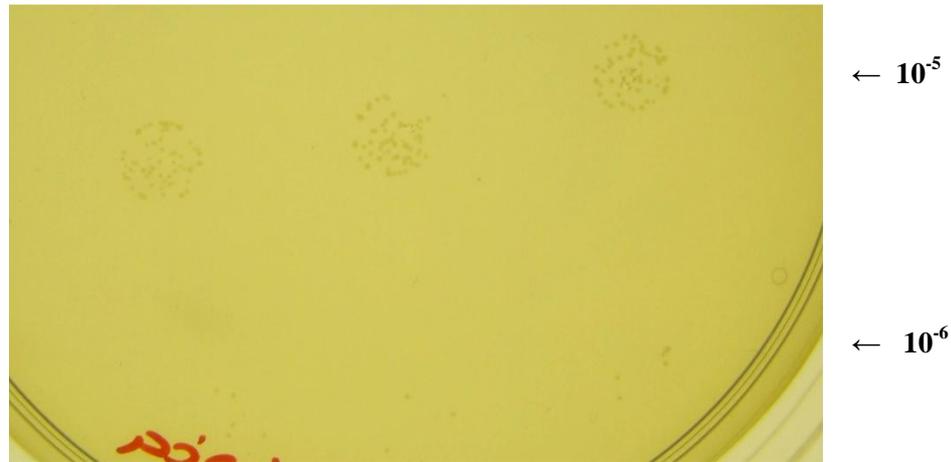


Figure 4-2 Colonies resulting from 10µl of a 10⁻⁵ and 10⁻⁶ dilution of bacterial suspension of unknown concentration using the Miles, Misra and Irwin method.

Sample 1 contained 5.83x10⁸ CFU per ml; sample 2 contained 7.33x10⁸ CFU per ml; giving an average of 6.6x10⁸ per ml for an OD of 0.81 (Table 4-2). The observed difference was probably due to variability in preparation of the two stock culture solutions.

Table 4-2 Miles, Misra and Irwin colony counts for three dilutions of two stock solutions (counts are number of colony forming units per 10µl)

Dilution	Sample 1 Room temperature			Sample 1 Incubator 37°C			Sample 2 Room temperature			Sample 2 Incubator 37°C			Average Sample 1	Average Sample 2
	4	4	6	8	10	3	10	8	6	7	9	4		
10 ⁻⁶	4	4	6	8	10	3	10	8	6	7	9	4	5.83	7.33
10 ⁻⁷	1	0	1	0	1	0	1	2	0	1	2	0	0.5	1
10 ⁻⁸	0	0	0	0	0	0	0	0	0	0	0	0	0	0

- ***Salmonella* spp. inoculum**

A single colony of *Salmonella* Bovismorbificans (as above) was transferred into a vial of LB broth and incubated at 37°C for 24h to prepare the inoculum for the *Salmonella* enrichment trial. After 24h, the suspension was pale yellow and turbid. The optical density of the broth was 0.86 when compared to LB broth alone. Given that initial dilutions had demonstrated that an OD of 0.81 corresponds to approximately 6.6x10⁸ bacteria/ml, the solution was diluted with LB broth to establish a concentration of 1.0x10⁸ bacteria/ml.

Subsequently, tenfold dilutions were prepared in LB broth to obtain concentrations of 10^7 , 10^6 , 10^5 , 10^4 , 10^3 and 10^2 bacteria/ml for the enrichment trial. Miles, Misra and Irwin plates were established as described previously using 10 μ l of each dilution to verify the accuracy of these dilutions.

- ***Salmonella* enrichment trial - Inoculation**

Given the difficulty of obtaining fresh seal faeces, fresh calf faeces were obtained as a comparable substitute. Four faecal samples were collected from 3 calves and 1 seal. Due to time constraints, the seal sample collected from the Scottish SPCA National Wildlife Centre had been maintained at 4°C for 5 days prior to the trial commencing; the calf faeces were collected from the Moredun Research Institute research farm from apparently healthy calves, undergoing no antimicrobial treatment, approximately 2 hours prior to the trial.

For each of the 4 faecal samples, 5 sterile Transwab® swabs (Medical Wire and Equipment Co. Ltd. (MWE), Corsham, UK) were heavily loaded with faeces and placed into individual glass vials containing 10ml selenite F broth (E and O labs, Bonnybridge, UK). Each of the 16 selenite broths were subsequently inoculated with either 1000, 100, 10 or ~1 *Salmonella* Bovismorbificans bacteria as follows and detailed in Table 4-3:

Concentration 1: 1000 = 100 μ l of dilution 10^4 bacteria/ml

Concentration 2: 100 = 100 μ l of dilution 10^3 bacteria/ml

Concentration 3: 10 = 10 μ l of dilution 10^3 bacteria/ml

Concentration 4: ~1 = 10 μ l of dilution 10^2 bacteria/ml

The remaining 5 vials were not inoculated with *Salmonella* spp. and served as controls. In addition, 4 selenite broths were inoculated with 1000, 100, 10 and 1 bacterium but no swab (Table 4-3). Vials were incubated for 24h at 37°C.

Table 4-3 Study design of *Salmonella* Bovismorbificans enrichment trial. Each vial of selenite F broth was incubated with calf faeces or seal scat and artificially spiked with 0 to 1000 CFU of *Salmonella* Bovismorbificans. Vials containing no calf faeces or seal scat acted as positive controls for each dilution of *Salmonella* Bovismorbificans.

Number of bacteria inoculated	1000	100	10	~1	0
<i>Salmonella</i> Bovismorbificans alone	1000 <i>Salmonella</i>	100 <i>Salmonella</i>	10 <i>Salmonella</i>	1 <i>Salmonella</i> bacterium	
Seal scat plus <i>Salmonella</i> Bovismorbificans	Seal scat + 1000 <i>Salmonella</i>	Seal scat + 100 <i>Salmonella</i>	Seal scat + 10 <i>Salmonella</i>	Seal scat + 1 <i>Salmonella</i> bacterium	Seal scat alone
Calf A faeces plus <i>Salmonella</i> Bovismorbificans	Calf A faeces + 1000 <i>Salmonella</i>	Calf A faeces + 100 <i>Salmonella</i>	Calf A faeces + 10 <i>Salmonella</i>	Calf A faeces + 1 <i>Salmonella</i> bacterium	Calf A faeces alone
Calf B faeces plus <i>Salmonella</i> Bovismorbificans	Calf B faeces + 1000 <i>Salmonella</i>	Calf B faeces + 100 <i>Salmonella</i>	Calf B faeces + 10 <i>Salmonella</i>	Calf B faeces + 1 <i>Salmonella</i> bacterium	Calf B faeces alone
Calf C faeces plus <i>Salmonella</i> Bovismorbificans	Calf C faeces + 1000 <i>Salmonella</i>	Calf C faeces + 100 <i>Salmonella</i>	Calf C faeces + 10 <i>Salmonella</i>	Calf C faeces + 1 <i>Salmonella</i> bacterium	Calf C faeces alone

After 24h incubation, 10µl of each selenite broth was directly plated out onto labelled brilliant green agar (BGA) (Oxoid, Basingstoke, UK) and nutrient agar (NA) plates (Oxoid). In parallel, 0.5ml of broth from the top quarter of the vial was placed into 3 cryovials, each containing 100µl sterile glycerol (Sigma-Aldrich, Gillingham, UK). The first vial was frozen at -80°C for 1 week; the second vial was frozen at -80°C for 16 months and the third vial was frozen indefinitely at -80°C. After 1 week and 16 months, respectively, vials were defrosted and 10µl of suspension plated onto labelled BGA and NA plates.

After 24h incubation at 37°C, semi-quantitative counts were carried out on BGA and NA plates with bacterial growth recorded as scant; 1+; 2+; 3+ or 4+. For BGA plates, a distinction was made between bright pink colonies (*Salmonella* spp.) and yellow colonies (lactose fermenting Gram negative bacteria). Semi-quantitative counts of each bacterial colony type were carried out. Colonies of *Proteus* spp. and pale pinkish colonies, unlikely to be *Salmonella* spp. were also counted and recorded as such. *Salmonella* spp. anti-sera was used to confirm isolation of

Salmonella enterica ssp. *enterica* serovar Bovismorbificans (O-6,7; O-8) on representative bright pink colonies and any unusual pinkish colonies.

4.2.2 Results

The Miles and Misra counts confirmed the concentration of the inoculum stock as 1×10^8 bacteria/ml.

Results of the enrichment and freezing trial are presented in Table 4-4. In all vials spiked with *Salmonella* spp. alone, florid bacterial growth was obtained from both non-frozen and frozen samples (Figure 4-3). Serum agglutination tests confirmed the bacteria as *Salmonella enterica* serovar Bovismorbificans. A heavy growth of *Salmonella* spp. was detected from all but one of the samples containing calf faeces spiked with *Salmonella* spp. (Figure 4-3). The sample from calf B spiked with a single *Salmonella* bacterium grew only lactose fermenting Gram negative bacteria after enrichment in both non-frozen and frozen samples.

The samples obtained from the seal scat gave very poor growth with very small numbers of pinkish colonies in all plates, whether the vials had been spiked with *Salmonella* or if seal scat alone was cultured.

Salmonella anti-sera was used to confirm isolation of *Salmonella* Bovismorbificans (O-6,7; O-8). The pale pink colonies detected in seal scat samples were Poly O negative; Poly H negative; O-6,7 negative and O-8 negative and are consequently unlikely to be *Salmonella* spp.. These pink colonies were sub-cultured onto BGA plates for further identification and incubated 24h at 37°C. Colonies remained variably pale pink to yellow and no agglutination was noted on poly O and poly H serum agglutination tests on these sub-cultured colonies. A single colony isolated from seal scat alone gave a pure culture of pink, medium sized colonies with discolouration of

the BGA plate in 24 hours at 37°C. Serum agglutination tests gave positive agglutination for poly-O; O-8 positive; poly-H positive and weak agglutination for O-6,7 antigens, suggesting that the seal scat may have harboured *Salmonella enterica* serovar Bovismorbificans, thus excluding this sample from the trial results due to the potential bias.

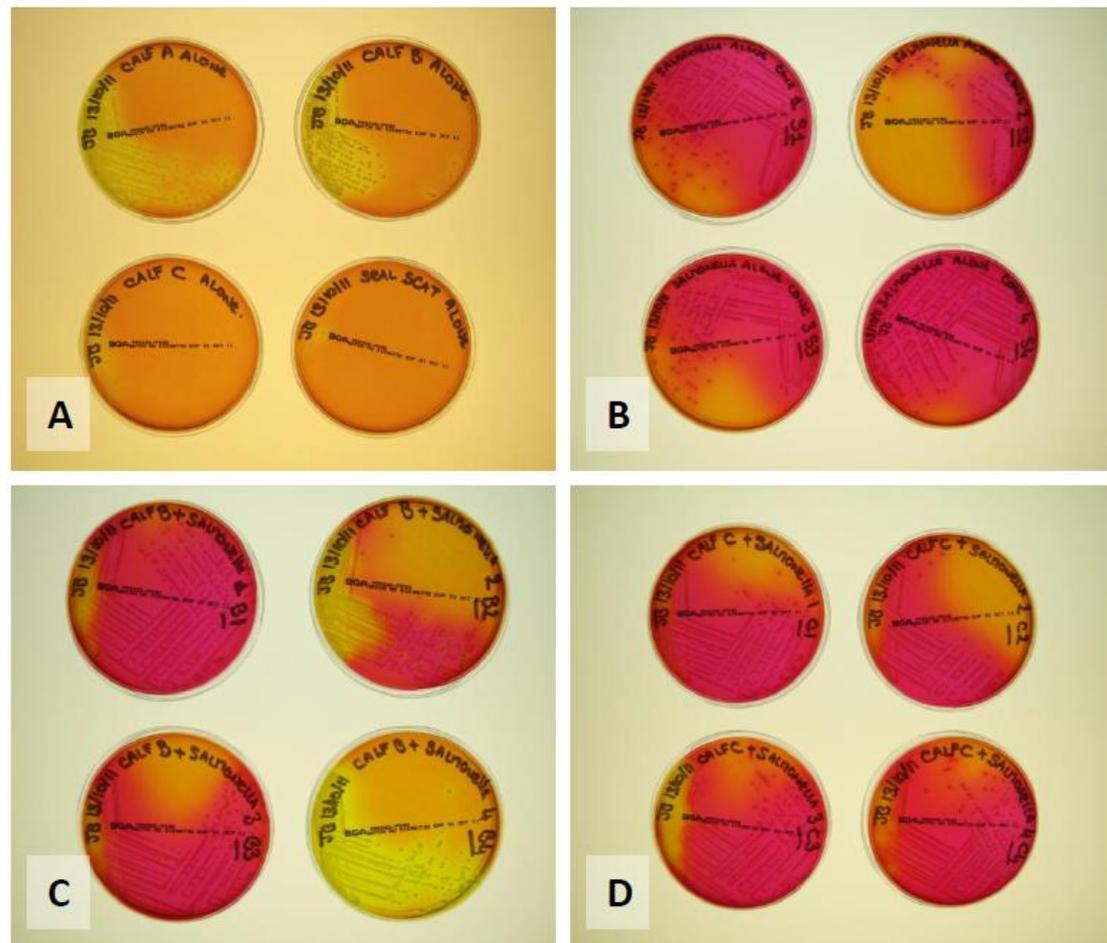


Figure 4-3 Bacterial growth obtained after enrichment trial on BGA plates. A: No bright pink colonies surrounded by intensely pink zones consistent with *Salmonella* spp. were identified in enriched samples containing only calf faeces or seal scat. Yellow colonies surrounded by intense yellow-green zones are consistent with lactose fermenting bacteria such as *E. coli*, *Klebsiella* or *Enterobacter* spp.. Occasional light pink colonies were present in the samples containing seal scat only. B: Dense growth of *Salmonella* spp. bacteria in selenite broth spiked with the four doses of *Salmonella* but no faeces or scat. C: Dense growth of *Salmonella* spp. bacteria in samples containing faeces from calf B, spiked with the four doses of *Salmonella*. Note the lack of *Salmonella* in the sample spiked with a single *Salmonella* bacterium, suggesting that the 10 μ l of suspension used to spike this broth contained no *Salmonella*. D: Dense growth of *Salmonella* spp. bacteria in samples containing faeces from calf C, spiked with the four doses of *Salmonella*.

Chapter 4 – *Salmonella* spp. in grey seals

Table 4-4 Results of *Salmonella* Bovismorbificans enrichment and freezing trial showing bacterial growth in non frozen cultures and in cultures frozen in glycerol at -80°C for 1 week and 16 months. Numbers represent semi-quantitative counts of bacterial growth recorded as scant; 1 (very light growth); 2 (light growth); 3 (moderate growth) or 4 (heavy growth). *Salmonella* were recognised as bright pink bacterial colonies on BGA plates with bright pink discolouration of the BGA. The presence of pinkish colonies was recorded in the absence of distinctive bright pink colonies. Lactose fermenting organisms were recognised as yellow colonies leading to bright yellow discolouration of the BGA.

Number of bacteria	Colonies	Calf A			Calf B			Calf C			Seal			<i>Salmonella</i> alone		
		Non frozen	Frozen 1 week	Frozen 16 months	Non frozen	Frozen 1 week	Frozen 16 months	Non frozen	Frozen 1 week	Frozen 16 months	Non frozen	Frozen 1 week	Frozen 16 months	Non frozen	Frozen 1 week	Frozen 16 months
1000	<i>Salmonella</i> spp.	3	4	4	4	4	4	3	4	4	10 pinkish colonies	8 pinkish colonies	8 pinkish colonies	3	4	3
	Lactose fermenters	1	1	2	0	1	1	0	1	2	1	1	1	0	0	0
100	<i>Salmonella</i> spp.	4	4	4	4	2	3	2	3	3	3 pinkish colonies	10-15 pinkish colonies	10-15 pinkish colonies	3	2	4
	Lactose fermenters	1	1	2	2	2	2	0	scant	1	2	2	2	0	0	0
10	<i>Salmonella</i> spp.	3	4	4	4	3	4	3	4	4	4 pinkish colonies	Single pinkish colony	Single pinkish colony	4	3	4
	Lactose fermenters	1	1	4	1	1	2	1	2	2	1	1	1	0	0	0
1	<i>Salmonella</i> spp.	4	4	4	0	0	0	2	4	4	2 pinkish colonies	Single pinkish colony	8 pinkish colonies	3	4	4
	Lactose fermenters	1	2	3	3	2	2	scant	1	2	1	1	1	0	0	0
0	<i>Salmonella</i> spp.	0	0	0	0	0	0	0	0	0	4 pinkish colonies	0	0			
	Lactose fermenters	2	2	2	2	2	2	2	1	1	1	1	1			

4.2.3 Discussion

Examination of vials containing *Salmonella* spp. alone demonstrated that the technique of enrichment with selenite broth allows detection of as few as 1 viable bacterial cell per sample. Furthermore, freezing for 1 week or 16 months does not affect this outcome.

The poor growth in all samples containing seal scat is of concern. No florid growth of *Salmonella* spp. was obtained in any of these samples following selenite broth enrichment. Instead several pinkish colonies were identified on BGA plates. Possible explanations for this may include prior antimicrobial treatment of this seal at the rehabilitation centre or a change in chemical composition of the scat due to prolonged storage. The treatment history of this seal pup was unknown but, if present, antimicrobial residues may have interfered with culture results. Further sub-culture and serum agglutination tests from pinkish colonies cultured from seal scat alone demonstrated positive agglutination for poly O; O-8 positive; poly H positive and weak agglutination for O-6,7, suggesting that the seal scat may have harboured *Salmonella enterica* serovar Bovismorbificans prior to experimental inoculation. Due to the poor growth of this sample and possibility of prior contamination with *S. enterica* serovar Bovismorbificans this sample was excluded from the trial results due to the potential bias.

The test samples containing only loaded faecal swabs from the three calves gave a low to moderate growth of lactose fermenting bacteria but no *Salmonella* spp. were identified suggesting that the samples were not contaminated with *Salmonella* spp. prior to the study. Vials containing a loaded faecal swab and spiked with 1000, 100 or 10 bacteria gave dense cultures of bright pink bacterial colonies, confirmed as *Salmonella enterica* serovar Bovismorbificans. Samples from calves A and C, spiked with a single *Salmonella* bacterium gave an abundant growth of *Salmonella*. Samples from calf B spiked with a single *Salmonella* bacterium grew no *Salmonella*. In reality, this broth was spiked with 10µl of a 10² bacteria per

ml suspension of *Salmonella*. Thus 10 μ l is likely to contain 1 or 0 bacteria. Given the lack of growth, it is most likely that the 10 μ l used to spike this broth contained no *Salmonella*, confirming the concentration of the initial broth.

In summary, the results of this small trial suggest that selenite enrichment of calf faecal swabs leads to a very high sensitivity (as low as 1 bacterium per vial) and that freezing at -80°C in 20% glycerol for up to 16 months does not affect the detection of *Salmonella enterica* serovar Bovismorbificans. Ideally this trial should have been repeated using fresh seal scat known to be untreated by antimicrobials and free of *Salmonella* spp.. However, time constraints due to the onset of the pupping season meant that the results of the calf faeces inoculation had to be considered sufficient to support the principle of enrichment in selenite F broth, followed by freezing at -80°C in glycerol for the subsequent field sampling process.

4.3 Materials and Methods

4.3.1 Animals and Samples

In Autumn 2011, rectal swabs were taken from 50 dead grey seal pups, 90 live, apparently healthy grey seal pups and 19 live yearling grey seals on their natal colony, the Isle of May (see Chapter 2). The live grey seal pups were sampled from three distinct sites on the Isle of May at three different time points (early, mid and late pupping season). Three sediment samples were also taken from each of two pupping locations within the colony (Tarbet slope and Rona Rocks).

Concurrently, rectal swabs were taken from 26 live grey seal pups found stranded along the Scottish coastline which had been transported to the Scottish SPCA National Wildlife Rescue Centre for rehabilitation. Pups were sampled within 24 hours of arrival at the rehabilitation centre and were not treated or co-habited until after sampling. Nine grey seal pups that

subsequently died or were euthanised on humane ground at the Scottish SPCA National Wildlife Rescue Centre were also sampled within 48h of death.

From both sites, rectal swabs were placed into Amies medium with charcoal (MWE, England) for bacterial culture. Swabs taken from live pups or at post mortem examination on the colony were processed within 12 hours of sampling; swabs taken at the rehabilitation centre incurred a postage delay of up to 2 days. A full post-mortem examination was performed on the 59 dead pups (colony n=50; rehabilitation n=9) (see Chapter 3). Samples of liver, spleen, brain and lung were systematically collected, frozen at -80°C and submitted to SAC Veterinary Consulting Services (SAC VCS) for bacteriology following routine methods (Quinn et al. 2011b). Formalin fixed samples of 26 organs were processed for histopathology using routine methods as described in Chapter 2.

For all animals the following data were systematically recorded: sex, sampling or stranding location (expressed as decimal degrees longitude and latitude), sampling date, weight (to the nearest 100g), girth at the axilla (to the nearest cm), length from nose to tail (to the nearest cm) and pup development stage code (as defined by Kovacs & Lavigne 1986).

4.3.2 Culture from swabs

The cotton tip of the faecal swab was aseptically severed, placed into a glass vial containing 10ml of Selenite F broth (buffered lactose peptone broth with added sodium biselenite) (E and O Laboratories, Bonnybridge, Scotland) and incubated aerobically at 37°C for 24h. One millilitre of the top third of the broth was collected, mixed with 200µl of sterile glycerol (Sigma-Aldrich) and stored in a cryovials at -80°C until required. At the end of the sampling period, cryovials were transported on dry ice to the SAC VCS laboratory for testing.

Samples in Selenite enrichment broth mixed with glycerol were removed from -80°C freezer and defrosted at room temperature for 30 minutes. Samples were gently mixed by inversion and 10µl of fluid was streaked out onto a Brilliant green agar plate (Oxoid) using a 10µl loop and incubated at 37°C for 18-24h. Each plate was visually assessed for the presence of non-lactose/sucrose-fermenting organisms, for lactose/sucrose-fermenting organisms and for individual small red colonies. Non-lactose/sucrose-fermenting organisms are recognised on BGA as red to pale pink, opaque colonies surrounded by bright red zones in the agar. These colonies are characteristic of *Salmonella* species (but not *Salmonella typhi*). Lactose/sucrose-fermenting organisms are recognised as yellow to greenish-yellow coloured colonies surrounded by intense yellow-green zones in the agar. These bacteria tend to be of the *Escherichia coli* or *Klebsiella/Enterobacter* group. Individual small red colonies may be *Proteus* and *Pseudomonas* spp.. The presence and abundance of each type of growth was recorded using a subjective scale of 0 to 3+ for both non-lactose fermenting organisms and lactose fermenting organisms, and a Present/Absent score for individual pinkish/red, ambiguous colonies.

Up to a maximum of 4 pink to red colonies were selected from each plate and streaked onto MacConkey agar plates (Oxoid). These subcultures were incubated for 18-24h at 37°C and resulting growth was assessed visually for purity. Colonies were examined using serum agglutination tests. Poly-O; Poly-H; O-6,7; O-8; O-4 and O-9 agglutinating serum were used (Remel Europe Ltd, Dartford, UK). A positive serum agglutination test result was recognised by clumping of latex beads when a suspension of bacterial isolate tested was mixed with the agglutinating serum and gently rocked from side to side. For each test, a negative control test was carried out using 0.85% saline solution in lieu of the test isolate suspension. For any plates where ambiguous pinkish colonies were recorded, a representative single pinkish colony was

selected and sub-cultured onto MacConkey agar and treated as a suspect *Salmonella* spp., as described above.

Following positive serological identification, an API 10S strip (Biomérieux UK Ltd, Basingstoke, UK) and a test purity plate were set up from a representative isolate from each animal. A single, clearly isolated colony of *Salmonella* spp. was selected and mixed with 5ml sterile saline solution. Ten microliters of the resulting suspension were inoculated onto Columbia agar with sheep blood (CSBA) (Oxoid) to assess purity of the culture and the remainder was used for the API 10S strip. If more than one serotype of *Salmonella* spp. was identified from the same animal, samples of each serotype were selected for further investigation.

API 10S strips were incubated for 16-24h at 37°C and read according to the manufacturer's instructions. For each *Salmonella* spp. confirmed by API 10S strip, serum agglutination tests were repeated on the purity plate culture to confirm purity and correlation with the original isolate. Each positive isolate was collected and stored on Microbank™ beads (Pro-Lab Diagnostics U.K., Neston, UK) at -80°C until required.

4.3.3 Identification of *Salmonella* isolates

Cultures were resuscitated onto CSBA plates and assessed visually for purity. A single colony was selected from each plate and results of serum agglutination tests re-confirmed using Poly-O; Poly-H; O-6,7 and O-4 agglutinating serum (Remel Europe Ltd). A single, clearly isolated colony was picked from each plate and transferred to sterile LB agar slopes. Slopes were incubated for 24h at 37°C prior to being submitted to the Scottish *Salmonella*, *Shigella* and *Clostridia difficile* reference laboratory (SSSCDRL) in Stobhill, Glasgow, where phage typing,

antimicrobial sensitivity tests, plasmid profiling, PFGE and MLVA were performed as detailed below.

- **Phage typing**

Phage typing of the *Salmonella* Typhimurium isolates was performed using the system established by Callow and subsequently extended by Anderson and Ward (Anderson et al. 1977; Callow 1959; Rabsch 2007). Briefly, definitive type (DT) designation in addition to the provisional type (PT) designation were established by detecting the presence of lysis in bacterial cultures induced by each of 39 specific bacteriophages.

- **Antimicrobial resistance**

Antimicrobial susceptibility was determined by breakpoint agar incorporation of antimicrobials for 14 agents, using a predetermined concentration of antimicrobial (Felmingham & Brown 2001). The breakpoint for ampicillin was 8µg/ml, chloramphenicol 1µg/ml, cefotaxime 1µg/ml, ciprofloxacin (low dose) 0.125µg/ml, ciprofloxacin (high dose) 1µg/ml, furazolidone 8µg/ml, gentamicin 4µg/ml, kanamycin 16µg/ml, nalidixic acid 16µg/ml, netilmicin 20µg/ml, spectinomycin 64µg/ml, streptomycin 16µg/ml, sulfamethoxazole 64µg/ml, tetracycline 8µg/ml and trimethoprim 2µg/ml. Suspensions of *Salmonella* were inoculated onto the surface of the plates; following incubation, if any growth was observed isolates were recorded as resistant to that antimicrobial at that concentration; in the absence of growth, isolates were recorded as sensitive.

- **Plasmid profiling Plasmid Profile Analysis (PPA)**

Plasmids were identified using the method of Kado and Liu (Kado & Liu 1981). Plasmid sizes were calculated by comparison with transconjugant *Escherichia coli* 39R861 and supercoiled ladder. Briefly, overnight cultures of each isolate were lysed by Kado and Liu lysis buffer (3% SDS in 50mM Tris, pH 12.6). The lysate was vigorously mixed with phenol:chloroform:isoamyl alcohol (25:24:1), centrifuged and the resulting supernatant was loaded onto a 0.7% agarose gel

and run for 3 hours at 100V. Following electrophoresis, the gel was stained with ethidium bromide, rinsed and visualised with GelDoc XR system (BioRad, Hemel Hempstead, UK).

- **Pulsed-field gel electrophoresis.**

PFGE for *S. Bovismorbificans* and *S. Typhimurium* was carried out as described (Ribot et al. 2006; Swaminathan et al. 2001). Briefly, agarose plugs were prepared using a pelleted overnight brain heart infusion (BHI) broth culture of each sample. Plugs for the control strains *Salmonella* Braenderup (strain # 267) and *Salmonella* Senftenberg (K99) were prepared in parallel (Hunter et al. 2005). Plugs were incubated overnight in a heated water bath (56°C) with ES buffer (0.5 M EDTA (Sigma-Aldrich, Gillingham, UK)–1% N-lauroyl sarcosine (Sigma-Aldrich) [pH 8.0 to 9.3]) and Proteinase K (Roche, Burgess Hill, UK) to lyse the cells. A 1mm slice of each plug was subsequently digested using the restriction enzyme XbaI (Invitrogen, Paisley, UK) and loaded into a 1% Pulsed Field Certified Agarose gel (BioRad) in 0.5 x Tris-Borate EDTA buffer (TBE) (Invitrogen). The gel was placed in a CHEF DR II electrophoresis tank (Bio-Rad), equilibrated in chilled TBE buffer and run at 6V/cm for 22 hours with an initial switch time of 2 seconds and a final switch time of 64 seconds. The gel was stained subsequently with ethidium bromide (Sigma-Aldrich), rinsed in distilled water and visualised under UV light using a BioRad GelDoc XR digital camera system.

As *S. Haifa* isolates have been found to be highly susceptible to genomic DNA degradation, PFGE for *S. Haifa* isolates was carried out as above with the addition of thiourea (VWR, Lutterworth, UK) to the electrophoresis buffer at a concentration of 200µM (Liesegang & Tschape 2002).

Images of the gels were analysed using the software Bionumerics Version 6.6 (Applied Maths, Kortrijk, Belgium) with optimization set at 1.3% and band tolerance at 1%. Relationships were determined by Dice correlation and Unweighted Pair Group Method with Arithmetic Mean

(UPGMA) clustering. Only restriction fragments of >33.3 kb were included in the analysis. Pulsotypes were compared to those stored in the SSSCDRL database and the PulseNET international database (<http://www.pulsenetinternational.org/>). The STYMXB nomenclature of PFGE profiles is based on the SalmGene classification (Peters et al. 2003), now superseded by PulseNet International. BmoX and HaiX designations are specific to the Scottish database and were employed when there were no matches for a profile in the PulseNet database.

- **MLVA - Multilocus variable number of tandem repeat analysis**

The three isolates of *S. Typhimurium* were further characterized using a sequence-based subtyping method, multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA), by following the standardized procedure established by PulseNet (ECDC 2011).

http://www.pulsenetinternational.org/SiteCollectionDocuments/mlva/PND15_MLVA_20Analysis_20Salm_20T_20Beckman.pdf.

Briefly, boiled-lysate suspensions of bacterial genomic DNA were submitted to a multiplex PCR reaction targeting 5 VNTR loci: STTR9, STTR5, STTR6, STTR10 and STTR3. Samples were subjected to denaturation (15 min at 95°C), followed by 25 cycles of 30 s at 94°C, 90s at 60°C, and 90 s at 72°C and a final extension step of 10 min at 72°C. PCR reactions were diluted with RNase free water, prepared for fragment analysis and loaded into a 3730 DNA analyzer with Pop-7 polymer (Applied Biosystems) for capillary electrophoresis. Electropherograms were subsequently analyzed using GenMapper software (Applied Biosystems) and MLVA alleles and patterns were assigned to each isolate. Fragment sizes for each VNTR locus were analyzed as categorical data in BioNumerics 6.6.

Fragment sizes for all loci were imported into Bionumerics 6.6, and allele numbers were assigned for each strain using pre-existing numbers stored in the Stobhill and Pulse Net database. All MLVA types were shown as fragment sizes in the following order: STTR9-

STTR5-STTR6-STTR10-STTR3. Data were analysed using Bionumerics 6.6 and compared to those stored in the SSSCDRL database. Minimum spanning trees were generated with Bionumerics 6.6 using categorical coefficient and UPGMA clustering.

4.3.4 Statistical analysis

Prevalence data were analysed using the R statistical software package (R Core Team 2013). For prevalence data, Fisher's exact tests were performed. Overall prevalence and odds ratios were calculated using a generalized linear model (GLM) with a binomial family and a logit link function. Site, sampling time, pup stage and the interactions between them were used as fixed explanatory factors.

4.4 Results

4.4.1 Identification

Culture of all 200 frozen samples of selenite enriched rectal swabs performed on BGA agar showed colonies of non-lactose fermenting organisms (bright pink colonies, typical of *Salmonella*) in 34 (17%) samples, lactose fermenting organisms (yellow colonies) in 113 samples (56.5%) and ambiguous pale pinkish colonies in 53 samples (26.5%). No growth was obtained in 38 (19%) samples. Up to 4 pink to red colonies or pale pinkish colonies were selected from each positive plate and sub-cultured on MacConkey agar plates (Oxoid). A total of 158 colonies were subbed from a total of 40 seals and 1 sediment sample.

Within the pink to red colonies (n=136), two isolates (from animals CL035 and CL053) did not show agglutination with poly-O or poly-H antigen, and were therefore excluded from the study. The remaining 134 colonies were both polyvalent-O, polyvalent-H positive, consistent with *Salmonella* spp.. Isolates were further sub-divided into those showing strong agglutination for

both 6,7-O and 8-O antigens (likely to be *Salmonella enterica* serovar Bovismorbificans) (n=119) and those negative for these antigens but positive for 4-O antigen (n=15) (likely to represent *S. Typhimurium*). All isolates were negative for 9-O antigen and only 8 isolates were positive for i-H antigen. These were 4-O positive. Thus 3 antigenic types were identified: 6,7-O, 8-O strains; 4-O i-H positive strains and 4-O i-H negative strains. A single isolate of each antigenic type was selected from each animal for further identification. A list of these isolates is provided in Table 4-5.

The majority (n=22) of pale pinkish colonies demonstrated no agglutination with Poly-O; Poly-H; O-6,7; O-8; O-4; O-9 and i-H antigen and were thus excluded from the study. However, pale pinkish colonies from samples taken from animals CL083, CL088, CD028, CD031 and CD033 showed agglutination with poly-O and poly-H antigens in all cases. Sample CL083 was negative for all other antigens tested and remained unidentified. Samples CL088 and CD031 were positive for O-6,7 and O-8 antigens also, strongly suggestive of *Salmonella enterica* serovar Bovismorbificans. Sample from CD028 and CD033 were both negative for O-6,7 and O-8 antigens, positive for O-4 antigen but CD028 was negative for i-H antigen whereas CD033 was positive. The antigenic profile of CD033 was most consistent with *Salmonella enterica* serovar Typhimurium, whereas that of CD028 remained undetermined.

API 10S strips were used to confirm the species of these suspected *Salmonella* spp. isolates. In two cases, the isolates were found to be different to *Salmonella*: CL083 was identified as *Citrobacter diversus* but the serum agglutination tests had previously been ambiguous. This isolate was deemed not to be a *Salmonella* spp. A015 (2) was identified as *Citrobacter freundii* but serum agglutination tests had initially been consistent with *Salmonella* Bovismorbificans. A colony was re-isolated from the original MacConkey plate and re-tested using the API 10S and purity plate. A015 (2) was subsequently confirmed by API 10S plate and serum agglutination tests to be *Salmonella* Bovismorbificans.

Following systematic culture of liver, lung, spleen and brain tissues from all 59 dead grey seal pups (see Chapter 3) *S. Bovismorbificans* was isolated from three pups: CD044 (lung, liver, spleen and brain), CD045 (liver, spleen and brain) and CD048 (liver and spleen). *S. Typhimurium* was isolated from the liver of pup CD051.

Serum agglutination tests, carried out at the Scottish *Shigella, Salmonella, and Clostridium difficile* reference laboratory (SSSCDRL), Stobhill, Glasgow confirmed the identity of all 33 isolates of *S. Bovismorbificans* and 4 isolates of *S. Typhimurium* (Table 4-5). Additionally, the two unidentified isolates, CD028 and A023, were both identified as *Salmonella enterica* ssp. *enterica* serotype Haifa.

Plasmid profiling of the 33 *Salmonella Bovismorbificans* strains revealed three distinct plasmid banding patterns (Table 4-5). Two isolates had only one plasmid of 90MDa; three isolates had two plasmid bands of 90MDa and 2.2MDa and the remaining 28 isolates had two plasmid bands of 90MDa and 2.4MDa. All isolates of *S. Bovismorbificans* were susceptible to all antimicrobials tested. Plasmid profiling and phage typing of the four *Salmonella Typhimurium* isolates revealed three distinct patterns (Table 4-5). Samples from animals CD033 and CD051 both had only one plasmid of 90MDa, were phage type 104 and were resistant to ampicillin (Amp), chloramphenicol (Chl), nalidixic acid (Nal), sulphonamide (Su), spectinomycin (Spe), streptomycin (Str), tetracycline (Tet) and low-dose ciprofloxacin (CipL) but were sensitive to all other antimicrobials tested including high-dose ciprofloxacin. The isolate from CD016, had a single plasmid of 90MDa, was identified as phage type 1 and was susceptible to all antimicrobials tested. The isolate from CL048 had two plasmid bands of 90MDa and 2.1MDa, was identified as phage type 41 and was susceptible to all antimicrobials tested. Both *Salmonella Haifa* isolates were found to be plasmid free and were susceptible to all antimicrobials tested.

Chapter 4 – *Salmonella* spp. in grey seals

Table 4-5 Origin, API-10S tests, serum agglutination characteristics, serovars, plasmid profiles, phage types and antimicrobial resistance characteristics of *Salmonella enterica* ssp. *enterica* isolates identified from grey seal rectal swabs, visceral organs and sediment in this study. + indicates a positive agglutination test; - indicates a negative agglutination test. Antimicrobials: A: Ampicillin, C: Chloramphenicol, Na: Nalidixic acid, Sp: Spectinomycin, St: Streptomycin, Su: Sulphamethoxazole, Tc: Tetracycline, CpL: Ciprofloxacin low dose.

Seal ID	Group		API-10S	8-O	6,7-O	4-O	9-O	r-H	i-H	Serovar	Plasmid Profile	Phage Type	Antimicrobial resistance	
R007	Stranded	Dead Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4	Sensitive	
A015	Stranded	Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4	Sensitive	
A018	Stranded	Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4	Sensitive	
A021	Stranded	Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4	Sensitive	
A022	Stranded	Live Pup	Rectal swab	6714	+	+	-	-	Weak	-	Bovismorbificans	90;2.4	Sensitive	
A023	Stranded	Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4	Sensitive	
A023	Stranded	Live Pup	Rectal swab	6714	-	-	+	-	-	-	Haifa	plasmid free	Sensitive	
A024	Stranded	Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4	Sensitive	
A026	Stranded	Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90	Sensitive	
CD015	Colony	Dead Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4	Sensitive	
CD016	Colony	Dead Pup	Rectal swab	6314	-	-	+	-	-	Weak	Typhimurium	90	1	Sensitive
CD028	Colony	Dead Pup	Rectal swab	6714	-	-	+	-	-	-	Haifa	plasmid free	Sensitive	
CD031	Colony	Dead Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90	Sensitive	
CD033	Colony	Dead Pup	Rectal swab	6714	-	-	+	-	-	+	Typhimurium	90	104	A, C, Na, Sp, St, Su, Tc, CpL
CD036	Colony	Dead Pup	Rectal swab	6714	+	+	-	-	Weak	-	Bovismorbificans	90;2.2	Sensitive	
CD038	Colony	Dead Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.2	Sensitive	
CD040	Colony	Dead Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4	Sensitive	
CD044	Colony	Dead Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4	Sensitive	

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Seal ID	Group		API-10S	8-O	6,7-O	4-O	9-O	r-H	i-H	Serovar	Plasmid Profile	Phage Type	Antimicrobial resistance
CD045	Colony Dead Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive
CD048	Colony Dead Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive
CD050	Colony Dead Pup	Rectal swab	6314	+	+	-	-	Weak	-	Bovismorbificans	90;2.2		Sensitive
CD051	Colony Dead Pup	Rectal swab	6714	-	-	+	-	-	+	Typhimurium	90	104	A, C, Na, Sp, St, Su, Tc, CpL
CL010	Colony Live Pup	Rectal swab	6314	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
CL036	Colony Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
CL038	Colony Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
CL039	Colony Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive
CL040	Colony Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
CL048	Colony Live Pup	Rectal swab	6714	-	-	+	-	-	Weak	Typhimurium	90; 2.1	41	Sensitive
CL062	Colony Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
CL063	Colony Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive
CL065	Colony Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive
CL068	Colony Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
CL069	Colony Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive
CL074	Colony Live Pup	Rectal swab	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive
CL078	Colony Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
CL085	Colony Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
CL088	Colony Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
CL090	Colony Live Pup	Rectal swab	6714	+	+	-	-	-	-	Bovismorbificans	90;2.4		Sensitive
RR1	Colony	Sediment	6714	+	+	-	-	weak	-	Bovismorbificans	90;2.4		Sensitive
CD051	Colony Dead Pup	Organs	6714	-	-	+	-	-	+	Typhimurium	90	104	A, C, Na, Sp, St, Su, Tc, CpL

Seal ID	Group		API-10S	8-O	6,7-O	4-O	9-O	r-H	i-H	Serovar	Plasmid Profile	Phage Type	Antimicrobial resistance
CD044	Colony Dead Pup	Organs	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive
CD045	Colony Dead Pup	Organs	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive
CD048	Colony Dead Pup	Organs	6714	+	+	-	-	+	-	Bovismorbificans	90;2.4		Sensitive

4.4.2 PFGE

PFGE of the 36 *S. Bovismorbificans* isolates (33 from rectal swabs; 1 from sediment and 3 from visceral organs) grouped them into two pulsotypes, both of which were identical to pulsotypes previously recorded by SSSCDRL. Thirty-five isolates were classed as pulsotype BmoX9 and a single isolate (or singleton) from seal pup A026 was classified as pulsotype BmoX4 (Figure 4-4). These two pulsotypes were closely related (~90% similarity) with a single band difference at either 763kbp or 683kpb for BmoX9 and BmoX4, respectively. Pup A026 was rescued from St Cyrus (~70km North of the Isle of May).

These two pulsotypes did not figure in the international database, the latter containing only 25 isolates of *S. Bovismorbificans* (from Italy, Finland, Denmark, Holland).

Within the SSSCDRL database, other BmoX9 pulsotypes have been recorded from faecal samples from cattle in Orkney and Caithness (10 isolates), from a sheep from Caithness and from visceral tissue samples four grey seal pups sampled in 2010 (Table 4-6). BmoX4 pulsotype had previously been recorded in visceral organs of a single grey seal in 2010 and in faecal samples of cattle from Dumfries and Galloway (5 isolates) in 2008 (Table 4-6). All strains of *S. Bovismorbificans* in the database were sensitive to all antimicrobials tested with the exception of a single BmoX12, multi-drug resistant strain isolated from a dog.

PFGE of the five *S. Typhimurium* isolates grouped the isolates into 3 distinct pulsotypes which correlated with the phage type described above (Figure 4-4). All three PFGE patterns were indistinguishable from isolates previously recorded in the SSSCDRL database. All three ST104s had identical PFGE profiles and were attributed to pulsotypes STYMXB.001; The ST41 was STYMXB.0029. The ST1 is STYMXB.0146 which is a PFGE pattern typical of phage type 41

isolates. Phage typing was repeated in light of this but the isolate was re-confirmed as phage type 1.

PFGE of the two isolates of *Salmonella* Haifa revealed that they shared the same pulsotype: HaiX9 (Figure 4-4). This PFGE pattern was indistinguishable from that of a previously reported *S.* Haifa isolate isolated from a 61 year old woman. The isolate had been submitted from the regional bacteriology lab in Fife, Scotland. Furthermore, this HaiX9 pulsotype was very similar to a pulsotype named “HaiX9+” isolated from two male human patients with a history of recent travel to Pakistan.

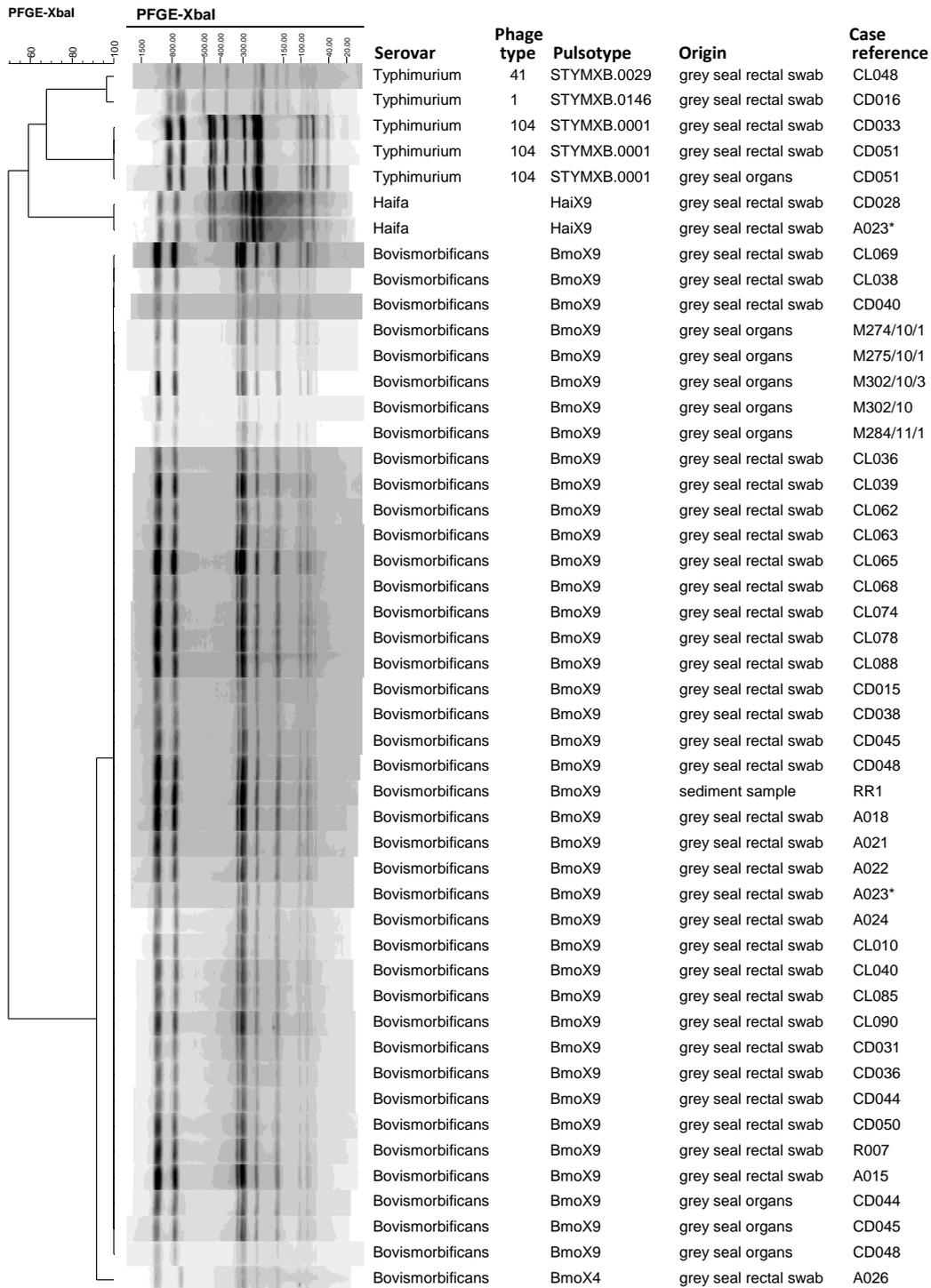


Figure 4-4 Dendrogram and PFGE patterns of 43 *Salmonella* spp. isolates found in grey seals and sediment in this study restricted with XbaI. Cluster analysis was performed with UPGMA using the Dice coefficient, a tolerance level of 1% and an optimisation level of 1.3%. For comparison, 5 isolates originating from grey seal pups sampled in 2010 are included in this diagram (M274/10/1, M275/10/1, M302/10/1, M302/10/3 and M284/11/1). Serovar, phage type, pulsotype, origin of the sample and case reference/animal reference are listed. The scale at the top indicates the similarity indices (in percentages) between isolates.*A co-culture of *S. Bovismorbificans* and *S. Typhimurium* was isolated from pup A023.

Table 4-6 List of the 19 PFGE XbaI patterns (pulsotypes) of *Salmonella* Bovismorbificans in the SSSCDRL database and corresponding host species. The two pulsotypes of *S. Bovismorbificans* found in grey seal pups in this study (BmoX4 and BmoX9) are shaded in grey. Isolates from the present study are not included in this table.

PFGE-XbaI-Pattern	Host species (number of isolates)	Additional comments
BmoX1	Human (1)	Recent travel: Kenya
BmoX2	Grey seal (1)	Nasal swab
BmoX3	Human (2)	
BmoX4	Grey seal (1), Bovine (5)	
BmoX5	Human (1)	
BmoX6	Human (1)	Recent foreign travel
BmoX7	Human (1)	
BmoX8	Human (1)	Recent travel: Lebanon
BmoX9	Grey seal (4), Cattle (10), Ovine (1)	NA
BmoX10	Human (1)	
BmoX11	Human (1)	
BmoX12	Human (2), Canine (2)	
BmoX13	Human (1)	Recent travel: Thailand
BmoX14	Human (1)	Recent foreign travel
BmoX15	Human (1)	Recent travel: Sri Lanka
BmoX16	Human (3)	
BmoX17	Human (1)	Recent travel: Malaysia
BmoX18	Human (1)	
BmoX19	Human (1)	
BmoX20	Human (1)	

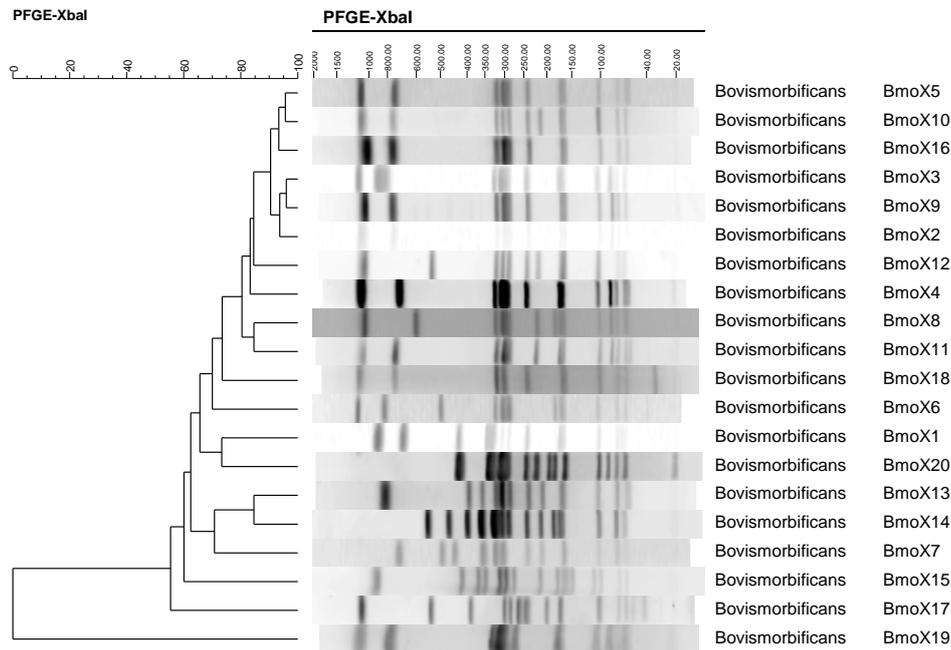


Figure 4-5 Dendrogram and PFGE patterns of the 19 *Salmonella* *Bovismorbificans* pulsotypes recorded in the SSSCDRL database restricted with XbaI. Cluster analysis was performed with UPGMA using the Dice coefficient, a tolerance level of 1% and an optimisation level of 1.3%. The scale at the top indicates the similarity indices (in percentages) between isolates.

4.4.3 MLVA

The five *S. Typhimurium* isolates were MLVA typed and 2 to 3 different alleles were found at each of the five MLVA loci (Figure 4-6). A minimum spanning tree was generated using the MLVA profiles of 921 *S. Typhimurium* isolates recorded in the SSSCDRL database sampled between 1990 and 2013 (Figure 4-7). Each node corresponds to an identical MLVA profile.

The three DT104 isolates had identical MLVA profiles (Figure 4-7). They also matched one other strain present in the SSSCDRL database: an isolate from a 3 year old girl from Edinburgh submitted in 2011. The closest related strains in this database were, an ovine *S. Typhimurium* DT104 from Caithness, showing only one change on the plasmid. The three DT104 isolates are

clustered within the majority of Scottish DT104s in the database with multiple bovine, ovine and human strains in close proximity to the seal DT104 isolates found in this study.

The DT1 isolate shared a MLVA pattern with one other *S. Typhimurium* isolate in the SSSCDDRL database: an environmental isolate of phage type DT 195 (Figure 4-7). However, the PFGE profiles of these two isolates were distinct. The MLVA pattern of this DT1 isolate was closely related to two DT40s differing only at one MLVA locus.

The DT41 isolate did not share a MLVA pattern with other *S. Typhimurium* isolates in the database but was closely linked to a DT2 (one MLVA locus difference) and two DT40 isolates (two MLVA loci difference) (Figure 4-7).

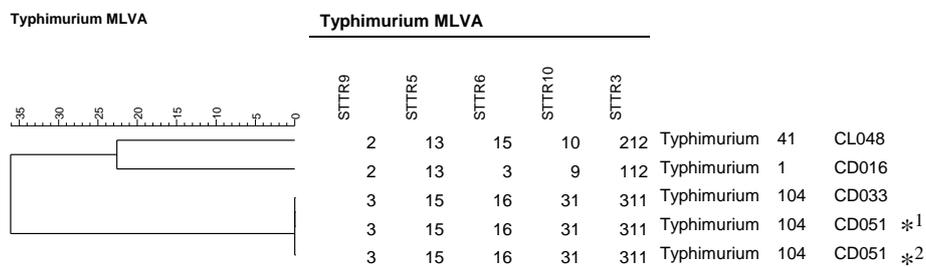


Figure 4-6 Dendrogram depicting MLVA types of the five isolates of *Salmonella Typhimurium* isolated from grey seals in this study. The scale at the top indicates the similarity indices (in percentages) between isolates determined using the Manhattan distance measure. *¹ Isolate originating from rectal swab of CD051 *² Isolate originating from liver of CD051.

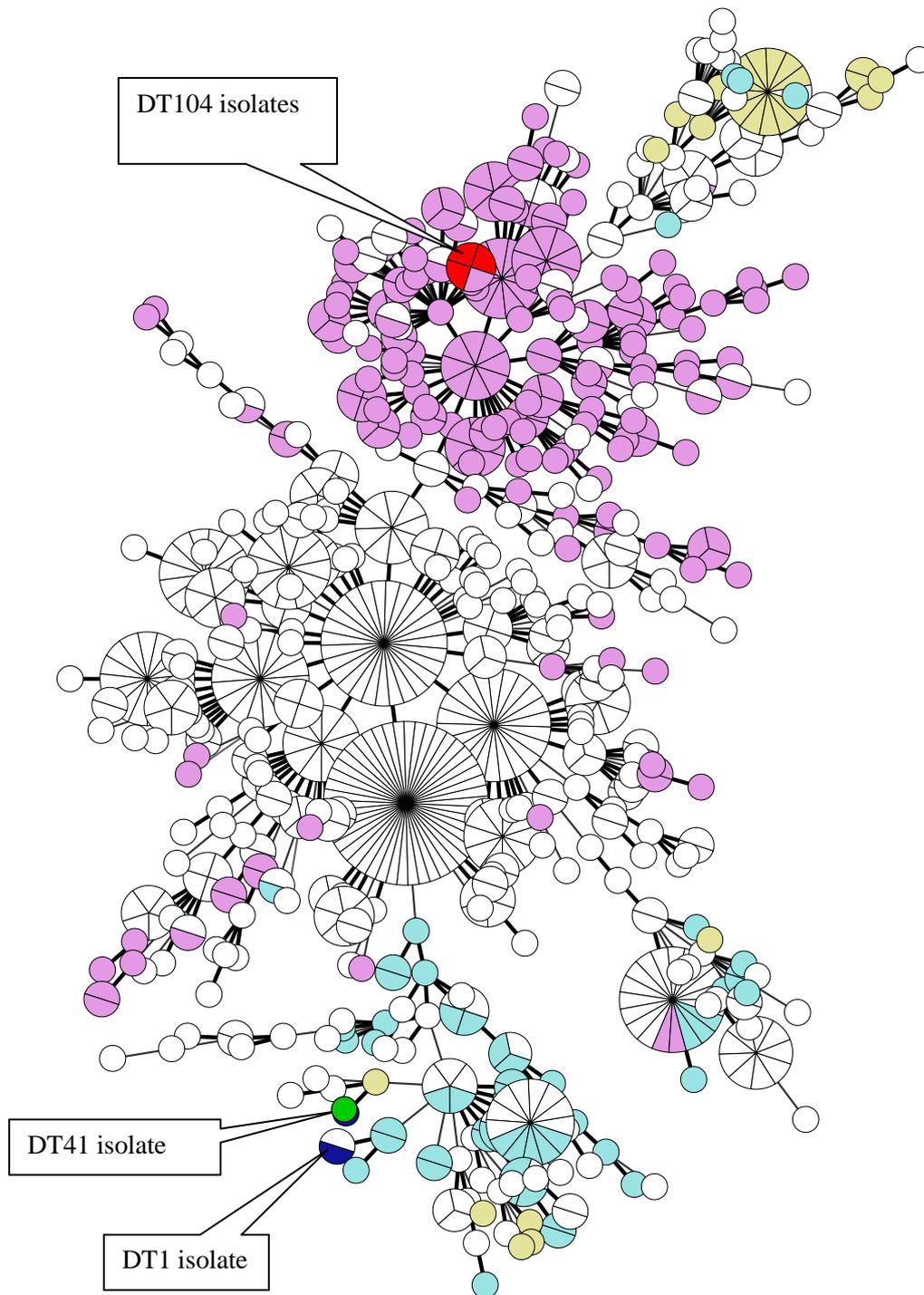


Figure 4-7 Minimum spanning tree established using MLVA profiles of 921 *Salmonella* Typhimurium isolates (1990 – 2013). Node size is proportional to the number of isolates belonging to each MLVA type. MLVA types differing at a single locus are separated by a thick branch; MLVA types differing at more than one locus are represented by a thin branch. MLVA types of isolate CD016 (DT1) is coloured in dark blue; isolate from CD048 (DT41) is coloured in green and the three DT104 isolates from this study are coloured in red. MLVA types for all previously recorded DT104 isolates in the SSSCDRL database are coloured in pink. MLVA types for all previously recorded DT56, DT40 or DT41 isolates in the SSSCDRL database are coloured in pale blue. MLVA types for all previously recorded DT2 isolates in the SSSCDRL database are coloured in pale yellow.

4.4.4 Epidemiology of *Salmonella*

The overall prevalence of *Salmonella enterica* ssp. *enterica* in all seals tested was 19% with a 21.1% prevalence of *Salmonella* in grey seal pups and 0% prevalence in grey seal yearlings (Table 4-7).

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Table 4-7 *Salmonella* prevalence in different groups. Numbers (%) animals positive for each *Salmonella* spp. within the group of interest; [95% confidence interval of percentage positive animals]; * one seal was positive for 2 isolates

	N Isolates	All seals combined (n=194)	Yearlings	Pups				Sediment	
			Isle of May Live (n=19)	All pups (n=175)	Isle of May		Rehabilitation		Isle of May (n=5)
					Dead (n=50)	Live (n=90)	Dead (n=9)	Live (n=26)	
<i>Salmonella</i> spp.	39	37 (19.1%)*	0 (0%)	37 (21.1%)	13 (26%)	16 (17.8%)	1 (11.1%)	7 (26.9%)	1
95% CI		[14.2, 25.2%]	[0, 16.8%]	[15.7, 27.8%]	[15.9, 39.6%]	[11.21,39.6%]	[0.5, 43.5%]	[13.7,46.1%]	
<i>Salmonella</i> Bovismorbificans	33	32 (16.5%)	0 (0%)	32 (18.3%)	9 (18%)	15 (16.6%)	1 (11.1%)	7 (26.9%)	1
95% CI		[11.9, 22.4%]	[0, 16.8%]	[13.3, 24.7%]	[9.7, 30.8%]	[10.4, 25.7%]	[0.5, 43.5%]	[13.7,46.1%]	
<i>Salmonella</i> Typhimurium	4	4 (2.1%)	0 (0%)	4 (2.3%)	3 (6%)	1 (1.1%)	0 (0%)	0 (0%)	0
95% CI		[0.8, 5.2%]	[0, 16.8%]	[0.8,5.7%]	[2.1, 16.2%]	[0.05, 6%]	[0, 29.9%]	[0, 12.9%]	
<i>Salmonella</i> Haifa	2	2 (1%)	0 (0%)	2 (1.1%)	1 (2%)	0 (0%)	0 (0%)	1 (3.8%)	0
95% CI		[0.3, 4.1%]	[0, 16.8%]	[0.3, 4.1%]	[0.1, 10.5%]	[0, 4.1%]	[0, 29.9%]	[0.02,18.9%]	

The prevalence of *S. Bovismorbificans* was higher in live seal pups arriving at the rehabilitation centre (26.9%) than in other groups. No significant differences were recorded between groups except between stranded live pups and live free-ranging yearlings (Fisher's exact test, $p=0.016$).

4.4.5 Geographical distribution

Although the positive cases of *Salmonella* spp. in the 26 pups submitted to the rehabilitation centre were predominantly found in pups rescued from the South-East region of Scotland (see Figure 4-8), there was no statistical evidence for increased prevalence in this area using a generalised linear model with latitude and longitude as fixed variables and presence of *Salmonella* spp. as the outcome variable.

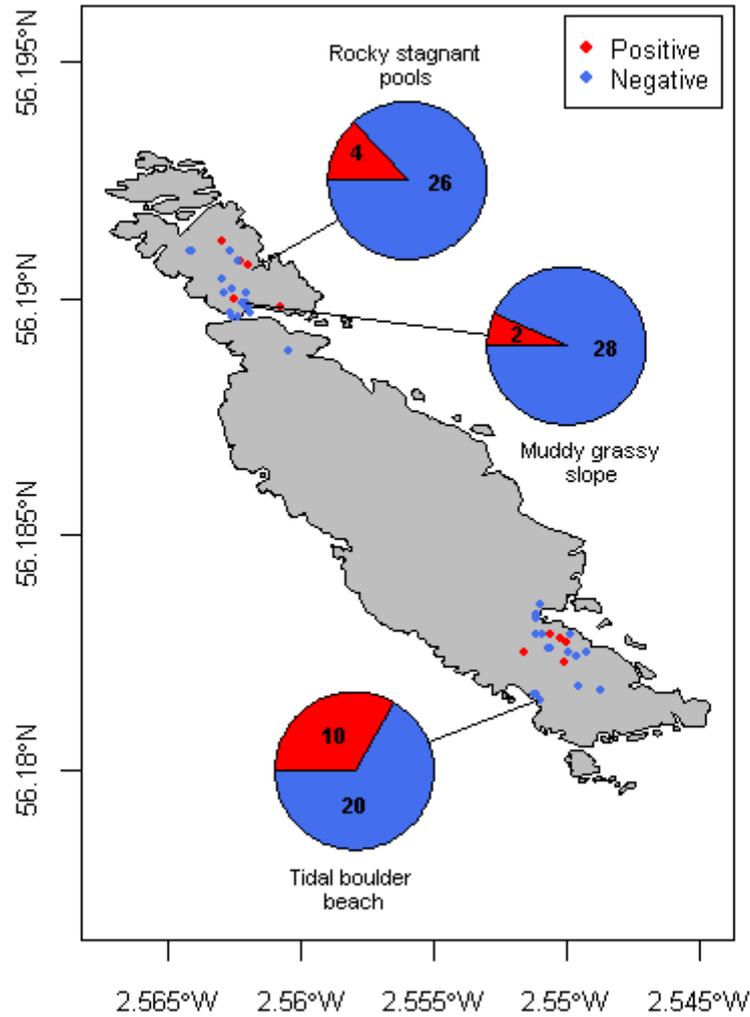


Figure 4-9 Map of locations of wild grey seals sampled for *Salmonella* spp. on the Isle of May. Individual dots represent locations in which dead pups were found; pie charts represent live seal pups at each of the three sampling sites (n=30 per sampling site). Red dot or proportion of pie: Isolation of *Salmonella* spp. on rectal swab; Blue dot or proportion of pie: No *Salmonella* isolated from rectal swab

4.4.6 Odds ratios and risk factors for the presence of *Salmonella* spp.

The prevalence of *S. Bovismorbificans* in live free-ranging grey seal pups was significantly higher at the tidal boulder beach site (33.3%) when compared to the grassy slope site (6.7%) ($p=0.021$; Fisher's exact test). The prevalence at the rocky stagnant pool site (13.3%) was substantially lower than that seen on the tidal boulder beach, with a difference approaching statistical significance ($p=0.057$; Fisher's exact test).

The tidal boulder beach of Pilgrim's haven consists of medium sized, volcanic greenstone boulders, interspersed with a small amount of coarse sand, delimited either side and at the back by steep olivine/dolerite (greenstone) cliffs. The beach is almost entirely covered by the incoming tide, leaving only a narrow strip of beach and small rocky caves as substrate for the pups at high tide. Throughout this twice daily process, pups are potentially exposed to sea water, but are also submitted to significant displacement and crowding.

Tarbet slope consists of a relatively open grassy slope, interspersed with small, rocky outcrops, which becomes increasingly muddy as the pupping season progresses. Movement of adult grey seals consists predominantly of regular commutes to small pools or to the sea at Rona cut, leading to occasional displacement and interactions of pups in this area with adult grey seals or pups.

Rona rocks, on the other hand, is an extensive, slightly sloping moonscape of undulating greenstone, dissected by numerous fault lines and forming numerous small freshwater pools which become heavily contaminated with seal faecal matter, detritus and dead pups over the course of the pupping season. Adult movements are restricted to commutes, often to the small, murky freshwater pools and occasionally to the sea. Pups tend to be situated on rocky to grassy

ground, often located within a depression or cleft and movements and interactions are relatively restricted.

For the live grey seal pups, pups sampled at the tidal boulder beach (Pilgrim's Haven) and stranded pups admitted to the rehabilitation centre were considered to have been exposed to seawater. The prevalence of *Salmonella* spp. was significantly higher in live pups exposed to sea water when compared to those not exposed to sea water ($p=0.004$, Fisher's exact test). Live pups having been exposed to sea water had a 3.92 times higher odds of carrying *Salmonella* spp. than those not exposed to sea water (glm, $p=0.01$).

There was a statistically significant increase in the prevalence of free-ranging live grey seal pups carrying *S. Bovismorbificans* on the Isle of May in the early (3.3%) and late (33.3%) sampling periods ($p=0.005$; Fisher's exact test). Fisher's exact test comparing the prevalence in early to mid pupping season showed no statistical significance.

Table 4-8 Categorical risk factors, using univariate analysis, for grey seals that are harbouring *Salmonella* spp.. N: number of animals per group; OR: odds ratio; 95% CI: 95% confidence interval; Sign: Statistical significance of results; NS: non-significant; *: p<0.05; **: p<0.01; ***: p<0.001

	Parameter	Group	N	Nb (%) positive	OR	95% CI	P-value	Sign.
All seals	Group	Free-ranging Live	90	16 (17.8%)				
		Free-ranging Dead	50	13 (26%)	1.63	0.71, 3.73	0.252	NS
		Stranded Live	26	7 (26.9%)	1.7	0.61, 4.73	0.306	NS
		Stranded Dead	9	1 (11.1%)	0.58	0.07, 4.95	0.617	NS
		Free ranging Yearlings	19	0 (0%)	0	0, Inf	0.986	NS
All pups	Weight (kg)	<12	19	3 (15.8%)				
		12 to 25	101	19 (18.8%)	1.23	0.33, 4.67	0.755	NS
		25 to 31	15	4 (26.7%)	1.94	0.36, 10.43	0.440	NS
		>31	40	11 (27.5%)	2.02	0.49, 8.33	0.329	NS
	Sex	Female	80	20 (25%)				
		Male	94	17 (18.1%)	0.66	0.32, 1.37	0.268	NS
	Colony	Stranded	26	8 (22.9%)				
		Free-ranging	140	29 (20.7%)	0.88	0.36, 2.14	0.781	NS
	Live/dead	Live	116	23 (19.8%)				
		Dead	59	14 (23.7%)	1.26	0.59, 2.67	0.551	NS
Pup age	Pup	170	37 (21.7%)					
	Stillborn	5	0 (0%)	0	0, Inf	0.989	NS	
All live pups	Exposure to sea water	No seawater	60	6 (10%)				
		Seawater	56	17 (30.4%)	3.92	1.42, 10.85	0.008	**
Free-ranging Live pups only	Sampling-time	Early	30	1 (3.3%)				
		Mid	30	5 (16.7%)	5.8	0.63, 53.01	0.119	NS
		Late	30	10 (33.3%)	14.5	1.72, 122.4	0.014	*
	Site	Tidal boulder beach	30	10 (33.3%)				
		Rocky pools	30	4 (13.3%)	0.31	0.08, 1.13	0.075	*
		Muddy grassy slope	30	2 (6.7%)	0.14	0.03, 0.72	0.019	*
	Pup stage	Stage 2	41	4 (12.2%)				
Stage 3		13	3 (23.1%)	2.16	0.44, 10.63	0.344	NS	
Moult		27	6 (22.2%)	2.06	0.56, 7.57	0.278	NS	
Adult coat		9	2 (22.2%)	2.06	0.33, 12.81	0.439	NS	

Table 4-9 Categorical risk factors, using univariate analysis, for grey seals that are harbouring *Salmonella* Bovismorbificans. (n=): number of animals per group; OR: odds ratio; 95% CI: 95% confidence interval; Sign: Statistical significance of results; NS: not significant; *: p<0.05; **: p<0.01; ***: p<0.001

	Parameter	Group	N	Nb (%) positive	OR	95% CI	P- value	Sign.
All seals	Group	Free-ranging Live	90	15 (16.7%)				
		Free-ranging Dead	50	9 (18%)	1.1	0.44, 2.73	0.244	NS
		Stranded Live	26	7 (26.9%)	1.84	0.66, 5.15	0.841	NS
		Stranded Dead	9	1 (11.1%)	0.63	0.07, 5.37	0.669	NS
		Free ranging Yearlings	19	0 (0%)	0	0, Inf	0.986	NS
		All pups	Weight (kg)	<12	19	3 (15.8%)		
12 to 25	101			17 (16.8%)	1.08	0.28, 4.12	0.91	NS
25 to 31	15			2 (13.3%)	0.82	0.12, 5.67	0.841	NS
>31	40			10 (25%)	1.78	0.43, 7.4	0.429	NS
Sex	Female		80	17 (21.3%)				
	Male		94	15 (16%)	0.7	0.33, 1.52	0.37	NS
Live/dead	Live		116	22 (19%)				
	Dead		59	10 (16.9%)	0.87	0.38, 1.99	0.744	NS
Pup age	Pup	170	37 (21.7%)					
	Stillborn	5	0 (0%)	0	0, Inf	0.989	NS	
All live pups	Exposure to sea water	No seawater	60	5 (8.3%)				
		Seawater	56	17 (30.4%)	4.79	1.63, 14.09	0.004	**
Free-ranging Live pups only	Sampling-time	Early	30	1 (3.3%)				
		Mid	30	4 (13.3%)	4.46	0.47, 42.51	0.194	NS
		Late	30	10 (33.3%)	14.5	1.72, 122.4	0.014	*
	Site	Tidal boulder beach	30	10 (33.3%)				
		Rocky pools	30	3 (10%)	0.22	0.05, 0.91	0.037	*
		Muddy grassy slope	30	2 (6.7%)	0.14	0.03, 0.72	0.019	*
	Pup stage	Stage 2	41	4 (9.75%)				
		Stage 3	13	3 (23.1%)	2.77	0.53, 14.48	0.224	NS
Moult		27	6 (22.2%)	2.64	0.67, 10.44	0.166	NS	
Adult coat		9	2 (22.2%)	2.64	0.4, 17.32	0.311	NS	

Using separate univariate analyses, two variables to be considered in the logistic model for live free-ranging grey seal pups were identified: site and sampling time. Pup stage and weight were not included in the logistic regression model due to the high correlation with sampling time. Stepwise selection showed that the most parsimonious logistic regression model consisted of site and sampling time with no interaction term. The results of the logistic regression model and the association between the outcome (*S. Bovismorbificans* isolation) and risk factors are shown in Table 4-10.

Table 4-10 Multivariate logistic regression analysis showing the factors associated with risk of carrying *Salmonella* *Bovismorbificans* in free-ranging live grey seal pups. S.E.: standard error or coefficient; 95% CI: 95% confidence interval; Sign: Statistical significance of results; NS: not significant; *: p<0.05; **: p<0.01; ***: p<0.001

Variable	Coefficient.	S.E.	Odds ratio (OR)	95% CI of OR	P-value	Sign.
Intercept	-2.48	1.05				
Sampling time						
Early	-	-	1	-	-	
Mid	1.60	1.18	4.46	0.47, 42.51	0.17	NS
Late	3.01	1.14	14.5	1.72, 122.4	0.008	**
Site						
Tidal boulder beach	-	-	1	-	-	
Rocky pools	-1.79	0.79	0.22	0.05, 0.91	0.024	*
Muddy grassy slope	-2.26	0.89	0.14	0.03, 0.72	0.011	*

When investigating *Salmonella* *Bovismorbificans* shedding in all pups, stranded or free-ranging, two variables using univariate analysis to be considered in the logistic model were identified: exposure to seawater and sampling time. Stepwise selection showed that the most parsimonious logistic regression model consisted of both exposure to seawater and sampling time with no interaction term. The results of the logistic regression model and the association between the outcome (*S. Bovismorbificans* isolation) and risk factors are shown in Table 4-11.

In summary, exposure to seawater and later date of sampling were associated with a higher risk of finding *Salmonella* in rectal swabs in free-ranging and stranded grey seal pups.

Table 4-11 Multivariate logistic regression analysis showing the factors associated with risk of carrying *Salmonella* Bovismorbificans in stranded and free-ranging live grey seal pups. S.E.: standard error or coefficient; 95% CI: 95% confidence interval; Sign: Statistical significance of results; NS: not significant; *: p<0.05; **: p<0.01; ***: p<0.00

Variable	Coefficient.	S.E.	Odds ratio (OR)	95% CI of OR	P-value	Sign.
Intercept	-4.93	1.15				
Sampling time						
Early	-	-	1	-	-	
Mid	1.69	1.16	4.75	0.51, 44.67	0.146	NS
Late	3.57	1.1	26.43	3.28, 242.7	0.001	**
Other	3.05	1.75	38	1.26, 1149	0.08	NS
Seawater						
No seawater	-	-	1	-	-	
Seawater	1.87	0.62	4.79	1.63, 14.09	0.003	**

- **Pathogenicity**

Salmonella spp. was cultured from rectal swabs of 13 of 59 pups presented for post-mortem examination (Figure 4-10). Four of these pups presented with a septicaemic spread of *Salmonella* spp. (bacteria present in more than one internal organ on culture).

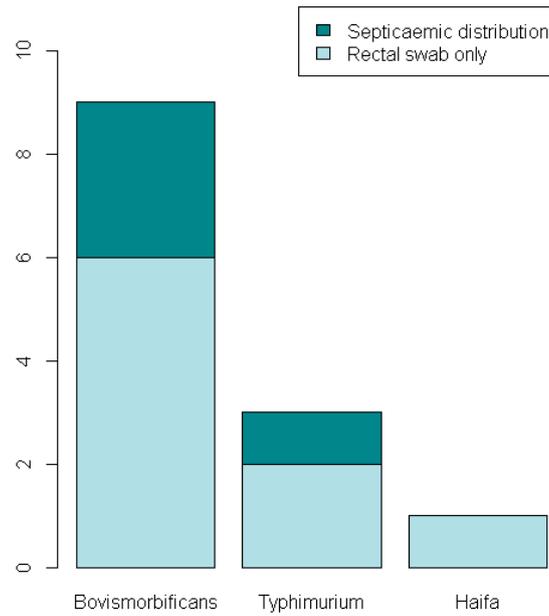


Figure 4-10 Number of dead grey seal pups positive for *Salmonella* spp. per serovar. Pale turquoise: Positive on rectal swab only; Dark turquoise: positive on rectal swab and tissue culture

Thus 33.3% (3/9) of dead pups positive for *Salmonella* Bovismorbificans on rectal swabs presented with a septicaemic spread of this bacteria; similarly 33.3% (1/3) of dead pups positive for *Salmonella* Typhimurium on rectal swab presented with a septicaemic spread of this bacteria.

Table 4-12 details the organs from which *Salmonella* spp. were isolated in each of the four cases, the most significant lesions/cause of death and any concurrent bacterial infections. Lesions associated with *Salmonella* Bovismorbificans septicaemia included omphalitis and peritonitis (Figure 4-11 and Figure 4-12). Concurrent bacterial infections were found in all 3 cases, with a noteworthy presence of *Streptococcus* spp. (*S. phocae* and *S. agalactiae*) and *Arcanobacterium phocae*. Lesions found in the seal pup presenting with *Salmonella* Typhimurium septicaemia included severe fibrino-necrotising interstitial pneumonia and

chronic-active encephalitis (Figure 4-13 and Figure 4-14). In all 4 cases, concurrent bacterial species isolated were from tissues (Table 4-12).

Table 4-12 Pathological findings, concurrent bacteriology results in 4 seals presenting septicaemic spread of *Salmonella* spp. Br: Brain, Li: Liver, Lu: Lung, Sp: Spleen

Seal ID	<i>Salmonella</i> serovar	Organs positive for <i>Salmonella</i> spp.	Main lesions / Cause of death	Concurrent bacterial infection
CD044	Bovismorbificans	Rectal swab, Liver, Lung, Spleen, Brain	Omphalitis, hepatitis, peritonitis and laryngeal necrosis	<i>Streptococcus phocae</i> (Lu, Br)
CD045	Bovismorbificans	Rectal swab, Liver, Spleen, Brain	Peritonitis, omphalitis, fibrino-necrotising broncho-interstitial pneumonia	<i>E. coli</i> (Lu); <i>Enterococcus faecalis</i> (Lu); <i>Arcanobacterium phocae</i> (Li, Sp); <i>Streptococcus phocae</i> (Li, Sp)
CD048	Bovismorbificans	Rectal swab, Liver, Spleen	Omphalitis, pneumonia, spinal abscess	<i>Actinomyces</i> sp. (Li, Lu, Sp); <i>Streptococcus agalactiae</i> (Sp); <i>Mycoplasma phocicerebrale</i> (Larynx); Mixed (Br, Larynx)
CD051	Typhimurium	Rectal swab, Liver, Brain	Fibrino-necrotising interstitial pneumonia	<i>Listeria monocytogenes</i> (Li); Mixed culture (Br)

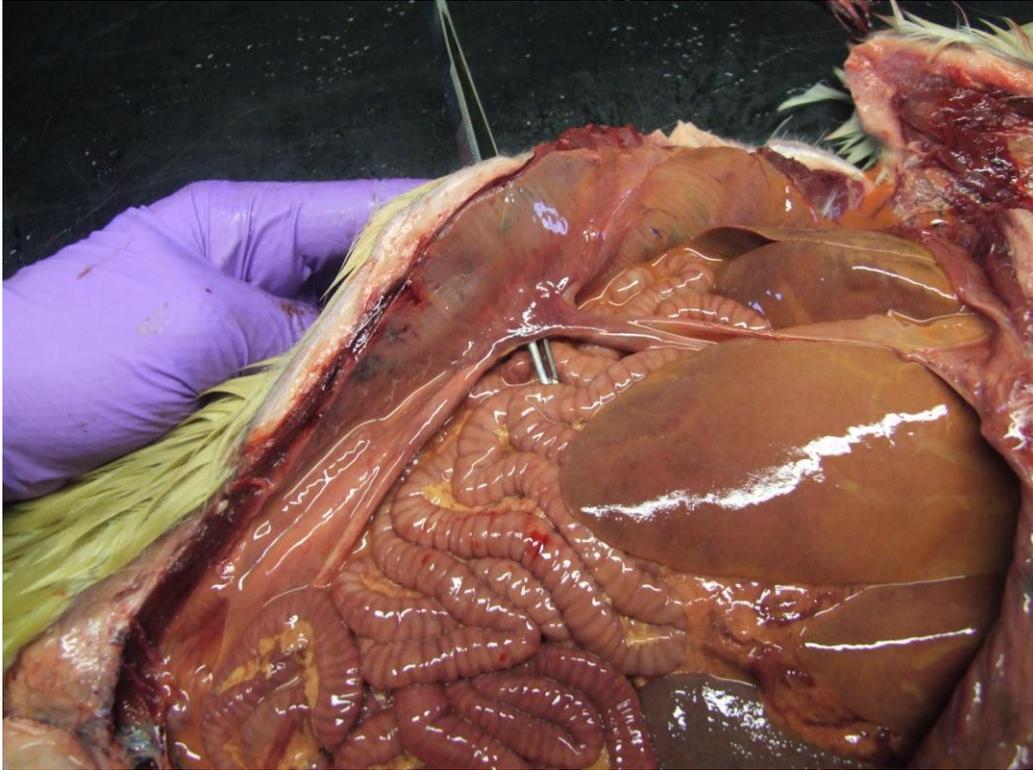


Figure 4-11 Suppurative omphalitis and peritonitis in a grey seal pup presenting septicaemic spread of *S. Bovismorbificans*. Forceps demonstrate direct communication between peritoneal cavity and exterior via a rupture in the urachus wall (case CD045)

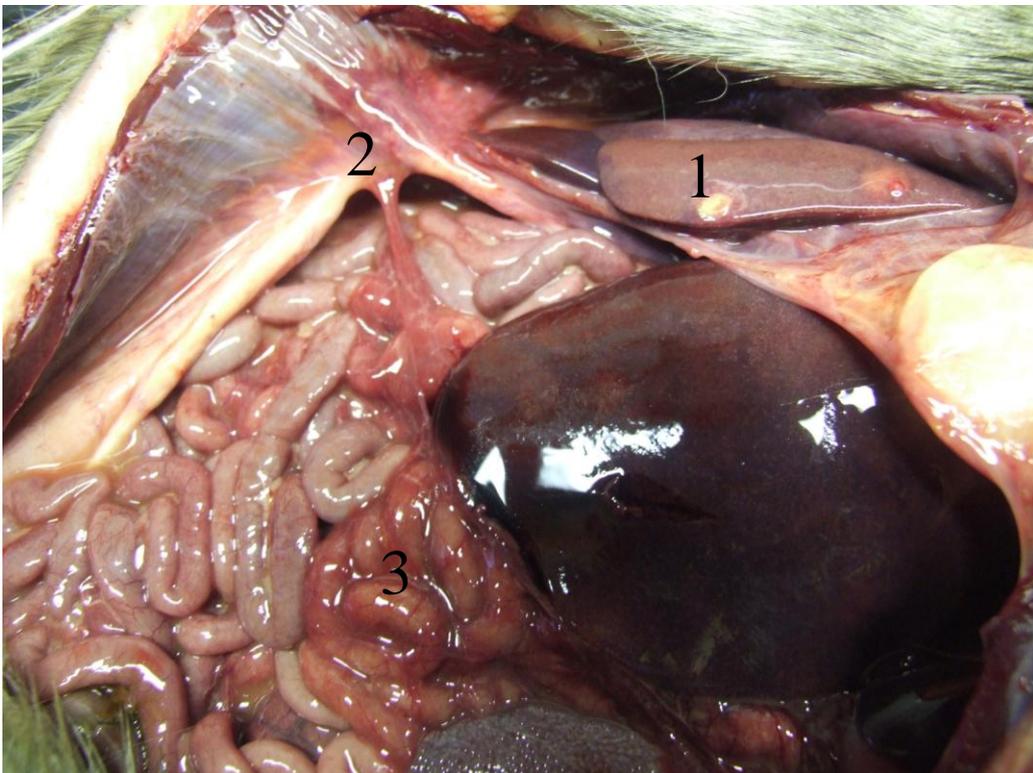


Figure 4-12 Necro-suppurative hepatitis (1), omphalitis (2) and peritonitis (3) in a grey seal pup presenting with a septicaemic spread of *S. Bovismorbificans* (Case CD048)

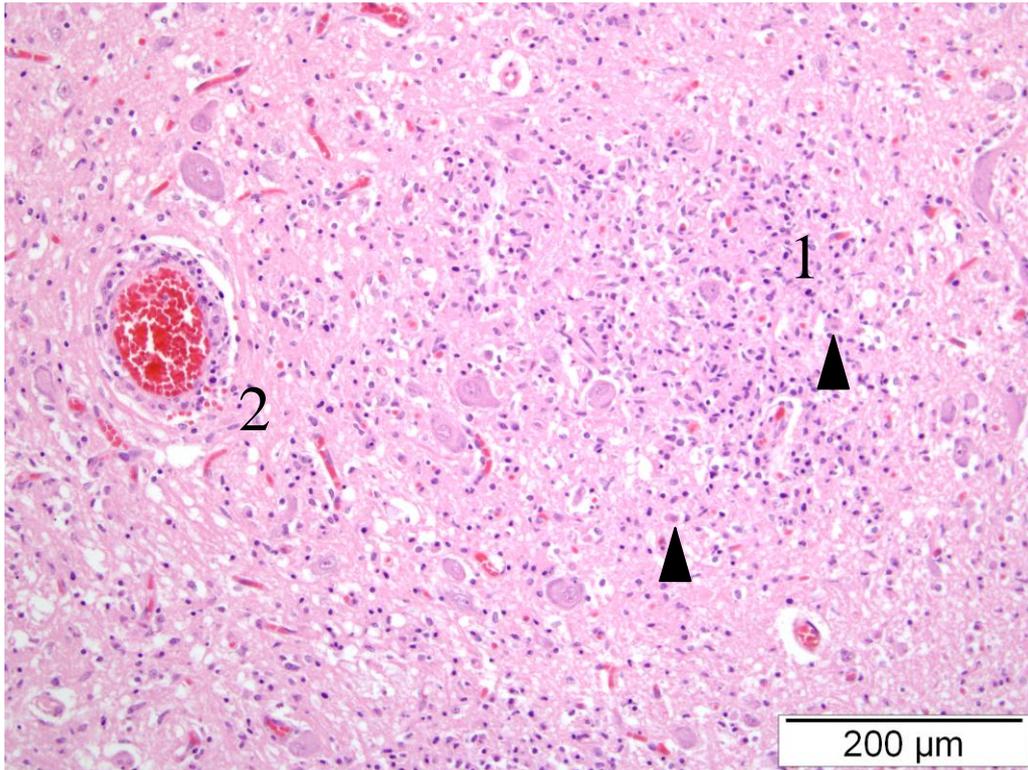


Figure 4-13 Brain. Chronic-active encephalitis (1) with neuronal necrosis (arrowheads) and lymphoplasmacytic perivascular cuffing (2) in a grey seal pup presenting with septicaemic spread of *S. Typhimurium* (Case CD051). H&E.

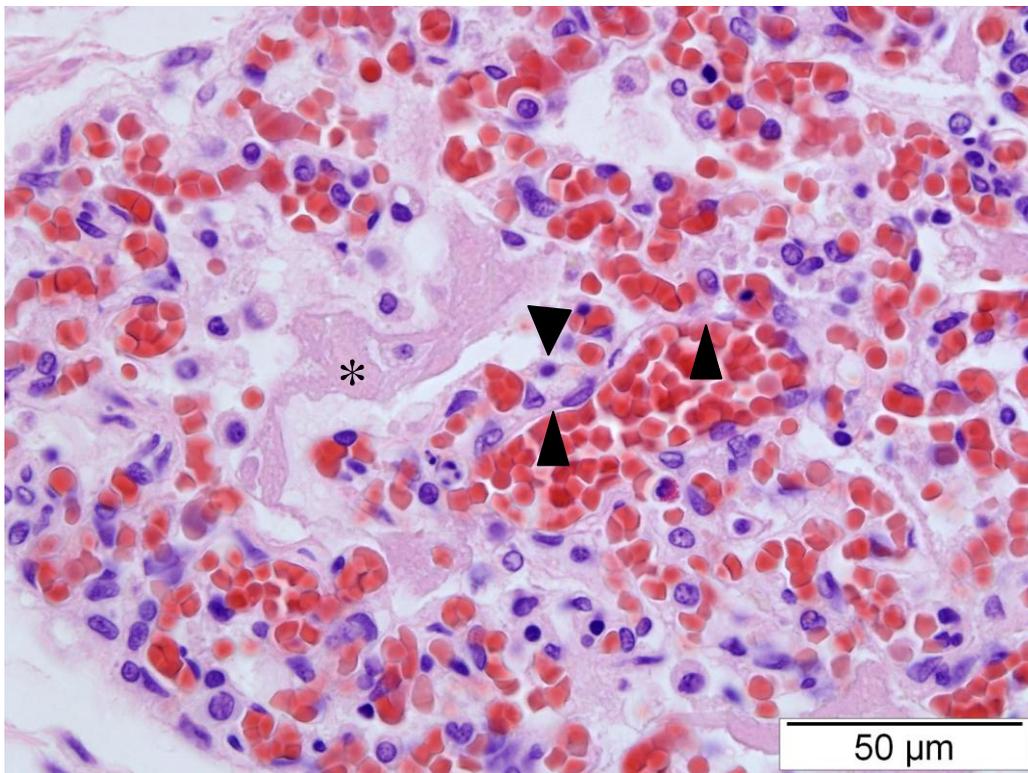


Figure 4-14 Lung. Fibrino-necrotising interstitial pneumonia in a grey seal pup presenting septicaemic spread of *S. Typhimurium* (Case CD051). Arrow head: Alveolar wall; *: fibrin. H&E.

4.5 Discussion

- **Prevalence**

The overall prevalence of *Salmonella* spp. in rectal swabs of live and dead grey seal pups in the present study was 21.1% with *Salmonella enterica* serovar Bovismorbificans, serovar Typhimurium and serovar Haifa the only three serotypes isolated. *Salmonellae* have previously been reported in Scottish grey seals with *S. Bovismorbificans* first identified in 1979 (Anderson et al. 1979) and *S. Typhimurium* DT104 in 1998 (Foster et al. 1998).

From a population perspective, the prevalence of *Salmonella* spp. in pinnipeds has been shown to range from 0% Northern fur seals in Alaska to 87% of free-ranging Northern Elephant seal pups in California (Baker et al. 1995; Fenwick et al. 2004; Gilmartin et al. 1979; Palmgren et al. 2000; Stoddard et al. 2005; Stoddard et al. 2008b; Vedros et al. 1982). The diversity of geographical location, host age, host species and season make it difficult to compare findings between studies. The prevalence of *Salmonella* in the present study is, however, comparable to the 11.8% prevalence found by Baker *et al.* (1995) in live grey seal pups on arrival at a rehabilitation centre in Norfolk.

Salmonella Newport has been the most consistently reported serovar in pinnipeds, recorded in California sea lions (Gilmartin et al. 1979; Stoddard et al. 2008b), Northern fur seals (Vedros et al. 1982), New Zealand fur seals (Fenwick et al. 2004), harbour seals (Baker et al. 1995) and Northern elephant seals (Stoddard et al. 2005). Other frequently reported serovars include *S. Typhimurium*, *S. Enteritidis* and *S. Heidelberg* (Gilmartin et al. 1979; Stoddard et al. 2008b; Vedros et al. 1982).

The lack of *Salmonella* spp. in samples from yearlings most likely indicates clearance of these bacteria from the gastrointestinal tract by one year of age. However, the possibility that the

yearlings sampled in this study had never been exposed to *Salmonella* spp. or that they were infected but simply not shedding the bacterium could not be excluded. This finding parallels that of a study in free-ranging California Sea lion pups sampled on the Channel Islands, California which had a 21% prevalence of *Salmonella* spp., compared to a 0% prevalence in free-ranging adult California sea lions in Puget Sound, Washington, USA. Unfortunately, it was not clear whether this was an effect of age or geographical location (Stoddard et al. 2008b). It is possible that there is negative association between *Salmonella* shedding and increasing host age, as suggested by Stoddard et al. (2008b). To investigate this hypothesis, individual animals could be repeatedly sampled.

The increased prevalence of a single pulsotype of *Salmonella* Bovismorbificans throughout the course of the breeding season suggests horizontal transmission of this pathogen within the breeding colony.

Grey seal pups exposed to seawater had a higher prevalence of *Salmonella* spp. when compared to those that had not been exposed to seawater, leading to the hypothesis that seawater may be a source of exposure to this pathogen. However it is important to note that other factors, such as stress, may be a factor as animals exposed to seawater (live stranded pups and pups on the tidal boulder beach) may have higher stress levels, in turn leading to decreased immune function, and ultimately, increased bacterial colonization/shedding. Studies in Northern Elephant Seals demonstrated a similarly higher prevalence of *Salmonella* spp. in stranded pups when compared to pups remaining on their natal beach (Stoddard et al. 2005).

Several studies have shown that *Salmonella* spp. can survive in seawater with little to no loss of total count (Hernroth et al. 2010; Morrison et al. 2011; Smith et al. 1994). *S. enterica* (unspecified serovar) was culturable on agar plates after 8 weeks of incubation in seawater at

both 8 and 18°C with little effect of temperature, UV exposure or nutrient loading on survival (Hernroth et al. 2010). Survival of *S. Newport* in brackish bay water was similar to survival in fresh water with a steady decline in both media over a 12 day period (Morrison et al. 2011). Although the survival of *Salmonella* spp. in seawater is relatively well established, there are multiple reports describing a decreased ability of *Salmonella* spp. to form colonies on solid media (Rozen & Belkin 2001; Smith et al. 1994) and the pathogenicity of *Salmonella* Typhimurium has been shown to decrease after 32 days exposure to nutrient poor sea water (Galdiereo et al. 1994).

Within the marine environment, *Salmonella* spp. have been most widely reported in association with filter feeding bivalve molluscs, such as mussels, clams and oysters, prone to contamination from faecal pathogens from sewage polluting their habitat (Wilson & Moore 1996). *Salmonella* spp. was cultured from 1.5-8% of market oysters in the USA (DePaola et al. 2010) and 8% of shellfish in the UK (Wilson & Moore 1996). More recent studies investigating the presence of *Salmonella* spp. in seawater and coastal areas (Martinez-Urtaza et al. 2004; Setti et al. 2009; Sudhanandh et al. 2012) have shown a wide range of *Salmonella enterica* serovars in coastal ecosystems in Spain, India and Morocco. *Salmonella* spp. was detected along the Spanish Galician coastline where individual pulsotypes of *S. Seftenberg* were shown to persist in the marine environment for up to 4 years, thought to be circulating within mussel populations (*Mytilus edulis*) (Martinez-Urtaza et al. 2004). In the same study, *Salmonella* was more frequently isolated from water column samples than from samples taken from the marine benthos (Martinez-Urtaza et al. 2004).

Although not statistically demonstrable, there appears to be a higher proportion of cases of *Salmonella* in the South East region of Scotland. It is tempting to speculate that this is a reflection of the proximity to large areas of urbanisation and high population density of the Forth and Tay estuaries or regions (see Figure 4-15), mirroring the higher prevalence of enteric

bacterial pathogens found in sea otters in California in more urbanised coastlines (Miller et al. 2002). The outcome of the present study is most likely limited by the relatively small sample size of pups tested on arrival at the rehabilitation centre and a larger, prospective study could be envisaged to investigate this hypothesis further.

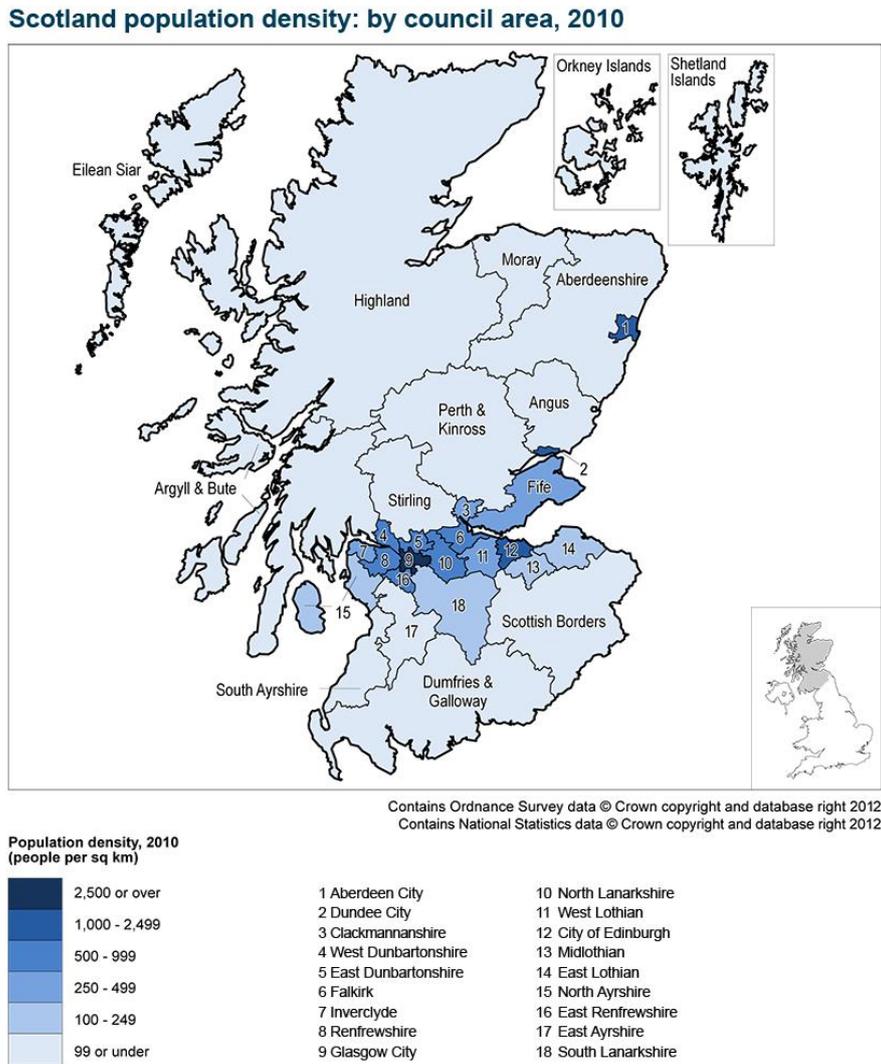


Figure 4-15 Human population density of Scotland by council area, 2010. Source: Office for national statistics.³

³ The human population density map of Scotland contains Ordnance Survey data (© Crown copyright and database rights 2012 Ordnance Survey 100019153) and National Statistics data (© Crown copyright and database right 2012)

- **One predominant serovar - Bovismorbificans**

Salmonella Bovismorbificans was present in all study groups of grey seal pups. *S.*

Bovismorbificans was first identified in grey seals in 1979 (Anderson et al. 1979) and has since been found in seals presenting with haemorrhagic gastroenteritis, focal hepatitis and sepsis (Anderson et al. 1979; Baker et al. 1980; Baker et al. 1995).

To the author's knowledge, *Salmonella* Bovismorbificans has not been reported in any other marine mammal species besides grey and harbour seals. *S.* Bovismorbificans was, however, isolated from sea birds including flesh footed shearwaters (*Puffinus carneipes*), common noddys (*Anous stolidus*), lesser noddys (*Anous tenuirostris*) and bridled terns (*Sterna anaethetus*), in Western Australia, but was not found in any of the marine mammals tested in the same study (Iveson et al. 2009). These authors suggest that exposure to sheep carcasses, discarded off-shore may be the source of this bacterium. *S.* Bovismorbificans has also been isolated from a European otter (*Lutra lutra*) and a guillemot (*Uria aalge*) (Baker et al. 1995).

PFGE typing of *Salmonella* Bovismorbificans isolates in the present study demonstrated only two, very similar pulsotypes. Comparison of these pulsotypes to those recorded in the SSSCDRL database showed indistinguishable strains isolated from cattle around Scotland.

The predominance of one pulsotype, BmoX9 in this study could indicate that this pulsotype is circulating, and possibly maintained in grey seal populations. Indeed, the isolation of *S.* Bovismorbificans from grey seals as early as the late 1970s could support the hypothesis that a strain of *S.* Bovismorbificans may be seal specific. The pulsotypes of these historical strains are not available, however.

To investigate whether there is evidence of co-evolution of seal and bovine strains of *S. Bovismorbificans*, suggesting regular exchange of the bacterium between these two host species; or whether there is evidence of genetic divergence between isolates, to suggest host adaptation, would require use of more advanced molecular techniques such as using whole genome sequencing (Mather et al. 2013).

It is noteworthy that, within Scotland, high densities of cattle farming are located on the East coast of Scotland and Orkney (Figure 4-16). A larger, prospective study of *S. Bovismorbificans* in grey seal populations would be warranted in order to investigate whether there is a spatial correlation between *S. Bovismorbificans* in grey seals and the cases of *S. Bovismorbificans* in cattle. There is no evidence of antimicrobial resistance in either bovine or seal strains.

Furthermore, extensive monitoring of fresh water outflow and coastal marine waters along the Scottish coastline could help investigate whether *Salmonella Bovismorbificans* infection of grey seals is due to a land-sea transfer of this pathogen.

Map showing "Total cattle

" in Scotland, 2011 (2km grid squares).

For area: 0,530000 to 575000,1220000.

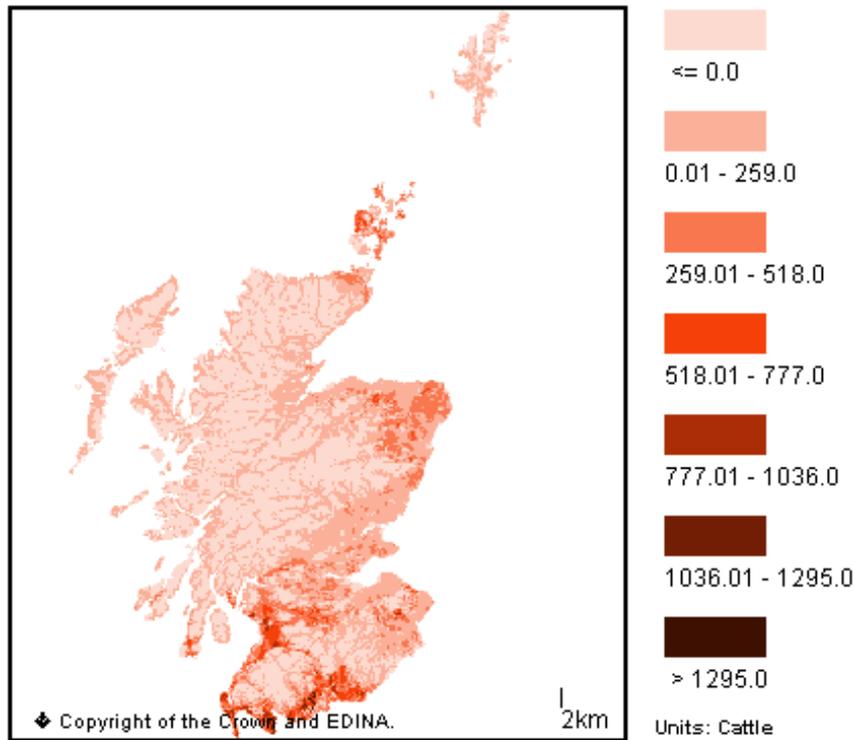


Figure 4-16 Map of cattle density in Scotland⁴. Data: number of cattle listed in Scotland in the 2011 Agricultural Census (data obtained through DigiMap EDINA software; <http://edina.ac.uk/agcensus/>) Units: cattle per 2 x 2km grid squares.

- ***S. Typhimurium***

Salmonella Typhimurium isolates were identified as phage types DT1, DT41 and DT104.

Molecular subtyping and comparison with the SSSCDRL database identified closely related strains isolated from human cases, livestock (cattle, sheep, reindeer, horses) and wild birds (garden birds, pheasants and gulls).

⁴ The grid square agricultural census data, as converted by Edinburgh University Data Library, are derived from data obtained for recognised geographies from the Department of Environment, Food and Rural Affairs (DEFRA), The Welsh Assembly Government, and The Scottish Government (formerly SEERAD), and are covered by Crown Copyright.

Reports of *S. Typhimurium* in marine mammals in UK waters are uncommon. *S. Typhimurium* phage type 49 was recorded in 3 (1.4%) of 208 free-ranging harbour seals in the Wash at a time when ST49 was a relatively common isolate in human laboratory submissions (Baker et al. 1995). This finding prompted debate as to whether this reflected exposure of harbour seals to untreated sewage, or whether *S. Typhimurium* was enzootic in harbour seal populations (Baker et al. 1995). A few years later, *S. Typhimurium* DT104 was described in a 12 week old stranded grey seal pup, known to have been born on the Isle of May (Foster et al. 1998).

S. Typhimurium DT41 was the most common strain isolated from gulls in study of *Salmonella enterica* from wild birds in Great Britain. In this study, both *S. Typhimurium* DT41 and DT40 were proposed as “wild bird” strains of *Salmonella*, recognised infrequently in farmed mammalian and bird species in Great Britain over the same time period (Pennycott et al. 2006). It is interesting to note that the DT1 identified in the present study is most closely related to other DT40 isolates rather than DT1 isolates when compared with MLVA, so more closely related to “wild bird” strains despite the unusual phage type. The phage typing was repeated in light of this result and was confirmed as DT1.

There is constant interaction between gulls, predominantly greater black backed gulls (*Larus marinus*) and lesser black-backed gulls (*Larus fuscus*), and seals on the Isle of May colony, often scavenging dead pups and placentas, so it is reasonable to suggest that there is some degree of transmission of *S. Typhimurium* between bird and seal populations. Furthermore, the Isle of May supports internationally important breeding populations of seabirds and over 250 species have been identified as either over-wintering or passage of birds on migration (SNH 2012).

- ***S. Haifa* – in animals and humans**

The isolation of *Salmonella* Haifa was unexpected. This pathogen was first described in 1950 in Israel, isolated from a 3 year old child with enteritis (Sapiro & Hirsch 1950). It has since been

isolated from food animals, slaughterhouse personnel and retail meat products worldwide (Tuchili et al. 1996; Zewdu & Cornelius 2009) with sporadic reports in the UK human population (Randall et al. 2004). The pathogenicity of this bacterium is largely unknown although fatal infection with *S. Haifa* was reported in a 76 year old man in Japan, along with concurrent infection of his 1 year old grandson (Kaibu et al. 2005).

Given that the *S. Haifa* isolated in the present study was indistinguishable on PFGE from that found in a human patient from Fife in 2009, so very close spatial and temporal distribution, it is tempting to speculate that one or the other species represented a source of contamination for the other.

- **Antimicrobial resistance**

Mirroring the study carried out by Stoddard *et al.* in elephant seals, very little antimicrobial resistance was found in the *Salmonellae* isolated, likely indicating a lack of selection pressure in wild animals (Stoddard et al. 2008b). In the present study, antimicrobial resistance was only recorded in the two isolates of *S. Typhimurium* DT104 which were highly multidrug resistant, as is characteristic of this phage type (Threlfall 2000).

- **Pathogenicity**

A third of dead pups harbouring *Salmonella* Bovismorbificans (3 of 9) and *Salmonella* Typhimurium (1 of 3) presented with a septicaemic spread of these bacteria. This finding confirms that both these isolates have the potential to cause septicaemia yet the trigger causing a switch between carriage and septicaemic spread is not clear. In particular the apparent association of *S. Bovismorbificans* with pathogens such as *Streptococcus phocae* and *Arcanobacterium phocae*, bacterial species highly correlated with the omphalitis- hepatitis-peritonitis complex described in Chapter 3, could indicate that septicaemic infection with *S.*

Bovismorbificans occurs via the umbilicus rather than following systemic spread of an intestinal infection. Identifying which lesions are specifically caused by *Salmonella* spp. is challenging given the presence of concurrent bacterial infection in each case. Further investigation into the significance of the concurrent bacterial infections and herpesvirus status of these pups is discussed in Chapter 6.

4.6 Conclusion

The presence of identical *Salmonella* *Bovismorbificans* isolates in cattle and *Salmonella* Typhimurium and *S. Haifa* isolates in human patients suggests a possible land-sea transfer of these enteric pathogens which warrants further investigation.

Chapter 5 Investigation of *Campylobacter* spp. in neonatal and juvenile Scottish grey seals

5.1 Introduction

5.1.1 Major human health problem

Campylobacter is a genus containing several species of zoonotic bacteria which can be found in the gastro-intestinal tract and genital tract of a wide range of host species worldwide (Moore et al. 2005; Quinn et al. 2011a). They are Gram negative, motile, spiral bacterial rods with fastidious growth requirements (Allos 2001; On et al. 1996). These microaerophilic organisms favour enriched culture media with decreased oxygen tension and increased carbon dioxide for growth (Quinn et al. 2011a). At the time of writing, the genus *Campylobacter* comprises 22 species and 8 subspecies (Debruyne et al. 2010). The thermophilic *Campylobacter jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *C. lari*, *C. coli*, *C. upsaliensis* and *C. helveticus* form a genetically close group of species which, besides *C. jejuni* subsp. *doylei*, are commonly isolated from the gastro-intestinal tract of humans and animals (On 2001). *Campylobacter fetus* tends to be more adapted to the bovine and ovine genital tract leading to reproductive disorders (On 2001; Quinn et al. 2011a).

Campylobacteriosis, caused primarily by the thermophilic *Campylobacter jejuni* spp. *jejuni* and *C. coli*, is the most common cause of bacterial gastroenteritis (food poisoning) in the UK, with over 500,000 community cases estimated to occur annually (Strachan & Forbes 2010; Tam et al. 2012). The bacteria colonise the gastro-intestinal tract resulting in profuse diarrhoea, malaise and vomiting (Young et al. 2007). Although generally a mild condition it can be fatal in very young infants, the elderly or immunosuppressed patients (Allos 2001). This pathogen has also been linked to development of a progressive ascending paralysis due to an acute immune-

mediated polyneuropathy known as Guillain-Barré syndrome (Moore et al. 2005; Revez et al. 2011; Taboada et al. 2007). This syndrome is poorly understood but is thought to be associated with a prior history of infection by specific strains of *C. jejuni* (Allos 2001). The number of cases of *Campylobacter* spp. infection in humans is seasonally variable with many more reported in the summer and autumn months (HPS) (Allos 2001; Humphrey et al. 2007).

Campylobacteriosis is therefore a major human health problem, costing the UK economy an estimated £500 million each year (Humphrey et al. 2007). Infection is largely attributed to consumption of livestock derived products, particularly poultry products (Sheppard et al. 2009a; Stabler et al. 2013; Wilson et al. 2008). Indeed this pathogen was recently the subject of a large Scottish government report (CaMPS/FSA Scotland 2009) which attributed human clinical cases to seven potential infection sources with chicken, sheep and cattle representing the major sources (31.5%, 12.1% and 12.9% respectively). Ingestion of undercooked meat and meat products is thought to be the major route of transmission but contaminated bathing waters and untreated drinking water are also sources of infection (Moore et al. 2005; Sheppard et al. 2009b; Wilson et al. 2008) (<http://www.who.int/mediacentre/factsheets/fs255/en/>). The role of wildlife and environmental reservoirs in the ecology of *Campylobacter* is poorly understood but wild birds have been implicated as sources of infection, along with consumption during recreational activities of water contaminated by wildlife. It is unclear, at present, whether wildlife represents a reservoir or a recipient of *Campylobacter* spp. (Brown et al. 2004; Hepworth et al. 2011).

5.1.2 Identification of *Campylobacter* spp.

Identification and typing of *Campylobacter* spp. is problematic but not only due to its fastidious growth requirements. Differentiation between *Campylobacter* spp. based on phenotypic tests has proven challenging (Steinhauserova et al. 2001) with substantial overlap of biochemical and tolerance test outcomes between species (On & Holmes 1991a; On & Holmes 1991b). In

particular one of the most discriminatory features of *C. jejuni* is their ability to hydrolyse hippurate, yet small proportions of hippurate-negative isolates have been found, making this assay unsatisfactory for the routine identification of thermophilic *Campylobacter* spp. (Lucey et al. 2004; Rautelin et al. 1999; Totten et al. 1987). More pertinent to the present investigation are phenotypic studies of novel species such as *C. insulaenigrae* which show substantial phenotypical overlap with previously described species and highlight the importance of including molecular methods of identification into the identification scheme for *Campylobacter* spp. (Stoddard et al. 2007).

PCR and sequence analysis of the 16S rRNA gene has emerged as the preferred genetic technique for bacterial species identification over and above traditional phenotypic characteristics as it has the advantage of being able to recognise novel pathogens and non-cultured bacteria (Clarridge 2004).

Multi-locus sequence typing (MLST) is currently the most widespread method of typing *Campylobacter* spp. and provides a highly reproducible, international nomenclature (Maiden et al. 1998). This process consists in determining the sequences of seven house-keeping genes, which differ between species of *Campylobacter*. The sequence at each locus is compared to that of known alleles and an allele number is assigned to each locus on that basis. From this combination of seven alleles a so-called “sequence type” or ST is determined and any sequence types sharing more than 4 alleles are considered to form a “clonal-complex” or CC (Dingle et al. 2001). Currently, *C. jejuni* and *C. coli* share the same, well established MLST scheme and database whereas for other species such as *C. lari* the MLST database is much less well established with few alleles and sequence types present in the database (<http://pubmlst.org/Campylobacter/>).

MLST is widely used in the field of *Campylobacter* research, but does have the limitation of only analysing sequence polymorphism in seven relatively small loci. Indeed, the *Campylobacter* genome is renowned for its instability, being prone to interspecies and intraspecies recombination (Sheppard et al. 2008; Suerbaum et al. 2001). Thus MLST is not able to identify differences in the accessory genome (see below) that may contribute to specialised host or niche adaptation. Comparative genome analyses may, in addition to MLST, be helpful to identify genetic markers predictive of the source of an infection or indicative of niche adaptation (Hepworth et al. 2011).

5.1.3 Concepts of core genome and pan genome

Within a population, the bacterial genome has a so-called “core genome”, containing the genes common to, and by extrapolation, deemed essential to that group or species of bacteria. All remaining genes, not shared by all, or a large proportion of the group, are described as accessory genes (Segerman 2012). Finally, within a species or group, the entire collection of core and accessory genes form the “pan genome”, almost like a library of possible genes known to the group or species (Medini et al. 2005). The pan genome of the *Campylobacter* genus continues to expand as new isolates are sequenced and added to databases. The “diversity” of a bacterial species implies that strains have many different accessory genes in various combinations, whereas the “plasticity” of a bacterial species implies gain or loss of specific genes resulting in phenotypic changes (Dorrell et al. 2005). Comparative genome analyses using microarrays and later whole genome sequencing point towards high levels of genome diversity but low levels of genome plasticity in *C. jejuni* therefore supporting the use of pan-genome comparisons (Dorrell et al. 2005; Hepworth et al. 2011).

The core genome of *Campylobacter* is therefore relatively stable while the pan-genome varies drastically between isolates due to the presence or absence of numerous accessory genes.

Analysis and comparison of the pan genome, looking at presence/absence and sequence variation of accessory genes will help understand the diversity and, possibly, host or niche adaptation of different *Campylobacter* species (Hepworth et al. 2011).

Analysis of core and accessory genes was initially developed using microarray technology, but with the rapid progress in next generation sequencing, and decreasing cost, comparison of whole genomes sequences is increasingly more accessible. A gene-by-gene approach to investigate the basis of phenotypes in bacteria such as *Campylobacter* has formed the basis of a web-accessible, curated database BIGSdb (Bacterial Isolate Genome Sequence Database) (Jolley & Maiden 2010; Sheppard et al. 2012).

5.1.4 *Campylobacter* in the marine environment and marine mammals

Reports of *Campylobacter* spp. are sparse in grey seals. *Campylobacter*-like organisms were seen on films from a grey seal pup with enteritis on the Monach Isles, Scotland, UK but these were not successfully cultured (Baker 1984). *Campylobacter fetus* spp. *fetus* was isolated from the gastric mucosa of a grey seal in a rehabilitation centre in Cornwall, UK, associated with mild gastritis (Nick Davison, pers. Comm. and Cornwall Wildlife Trust, Marine Strandings in Cornwall and the Isles of Scilly 2009).

However, a growing number of studies have shown that *Campylobacter* spp. can infect and be isolated from other marine mammal species such as Stellar sea lions (*Eumetopias jubatus*) in Alaska, USA (Carrasco et al. 2011), northern elephant seal pups (*Mirounga angustirostris*) in California, USA (Stoddard et al. 2005; Stoddard et al. 2007), Antarctic fur seals (*Arctocephalus gazella*) in Antarctica (Garcia-Pena et al. 2010), harbour seals (*Phoca vitulina*) and a harbour porpoise (*Phocoena phocoena*) in Scotland, UK (Foster et al. 2004), southern sea otters in California, USA (*Enhydra lutris nereis*) (Oates et al. 2012) and a potentially novel species of

Campylobacter from the oral cavity of captive south American bottlenose dolphins (*Tursiops truncatus*) in Argentina. This pathogen has also been detected within the marine environment, in 5% of beach sand samples in California, USA (Yamahara et al. 2012), 46-50% of sand samples from UK beaches (Bolton et al. 1999) and a wide range of shellfish and seabird species in the UK and worldwide (Endtz et al. 1997; Obiri-Danso & Jones 1999; Skirrow & Benjamin 1980; Wilson & Moore 1996).

A potentially marine mammal specific species of *Campylobacter*, *C. insulaenigrae* was first described in Scotland in a harbour porpoise and 3 harbour seals (Foster et al. 2004).

Characteristics of this species include failure to grow at 42°C and poor tolerance for salt in culture media. This bacterium has since been identified in several other marine mammals: northern elephant seals, Antarctic fur seals and a South American sea lion (*Otaria flavescens*) and has shown a much wider phenotypical variation than first described (Garcia-Pena et al. 2010; Gonzalez et al. 2011; Stoddard et al. 2007). Stoddard (2007) reclassified 45 presumptive *C. lari* strains and 26 unidentified strains of *Campylobacter* as *C. insulaenigrae*. This wide phenotypic variability emphasises the need to include molecular methods, such as MLST, in species determination of *Campylobacter* spp. (Stoddard et al. 2007). Additionally, *C. insulaenigrae* was described in a human patient who presented with gastroenteritis and septicemia. This patient had had no contact with marine animals but was immunocompromised due to hepatic and renal failure (Chua et al. 2007).

C. jejuni has been associated with chronic diarrhoea in dogs (Brown et al. 1999) and experimental infections of puppies have led to mild enteric disease and superficial colonisation (Macartney et al. 1988). However, some species, including cats, dogs and humans, can carry *Campylobacter* asymptotically with no apparent clinical effects (Fleming 1983; Glass et al.

1983; Nair et al. 1985). The actual pathogenicity of *Campylobacter* spp. in marine mammals is poorly explored and therefore warrants further investigation.

5.1.5 Aim of the study

The aim of the present study was firstly to determine whether *Campylobacter* spp. was present in neonatal and juvenile grey seals and, if so, to assess the prevalence and species distribution in live and dead, free-ranging grey seal pups and yearlings and in stranded live and dead pups.

To help elucidate the origin of seal isolates and their relationship with known terrestrial and human isolates, extensive phenotyping and whole genome sequencing of bacterial isolates were performed. From the genome sequences, 16s rRNA sequence analysis, MLST and pan-genome analysis were carried out. In order to ascertain whether these were likely to be seal-specific isolates, circulating in a relatively closed ecosystem, diverging from other isolates or whether they were intrinsically linked with environmental, human and terrestrial animal isolates, pointing towards a degree of environmental pollution, genomes and MLST sequences were compared to known isolates of *Campylobacter* from known human clinical cases and other, potential source species using the BIGSdb database. Localisation of *Campylobacter* spp. within the tissues of dead pups was also carried out using immunohistochemical methods and the putative pathogenicity of *Campylobacter* was explored using routine histopathological examination.

Prior to field sampling, a small trial investigated whether storing swabs at various would be a suitable alternative to traditional culture techniques (see below).

5.2 Materials and Methods

5.2.1 Preliminary trial

Given the logistical constraints of culturing *Campylobacter* spp. in a field laboratory, combined with the highly fastidious nature of this organism, swabs were subjected to freezing at -80°C or medium to long term refrigerated storage, at 4°C, to determine the effect of delayed sample processing and freezing on the ability to culture *Campylobacter* spp.

- **Methods**

Cultures of *Campylobacter jejuni*, kindly donated by Geoff Foster, SRUC Inverness, were resuscitated from Microbank beads (Prob-Lab diagnostics, Wirral, UK) onto charcoal cefoperazone desoxycholate agar (CCDA) *Campylobacter* blood-free selective agar plates (Oxoid Ltd, Basingstoke, UK) and incubated for 48h at 37°C under microaerophilic environment using the Campygen system (Oxoid). Faecal samples were collected from a healthy bovine calf and a grey seal (as described in section 4.2.2). Approximately 1g of each faecal sample was placed into individual sterile petri dishes and mixed with 3 colonies of *C. jejuni* from the CCDA agar plate. Three sterile Transwab® swabs (Medical Wire and Equipment Co. Ltd. (MWE), Corsham, UK) were heavily loaded with each of the *C. jejuni* spiked faeces and placed into Amies with charcoal transport media. Three additional sterile Transwabs were loaded with 3 colonies of *C. jejuni* directly taken from the CCDA plate and placed into Amies with charcoal transport media. One swab from each sample group was immediately frozen at -80°C and the second swab from each group was refrigerated at 4°C. The remaining 3 swabs were plated immediately onto CCDA agar (see Table 5-1) and incubated at 37°C for 48h under microaerophilic environment. After 8 days, frozen and refrigerated swabs were slowly brought to room temperature and plated onto CCDA and Skirrow's agar plates (E and O laboratories, Bonnybridge, UK). Plates were incubated at 37°C for 48h under microaerophilic environment and examined for growth.

Table 5-1 Swabs prepared to assess the effect of delayed sample processing and freezing on the ability to culture *Campylobacter* spp.

Swab	No treatment	Frozen -80°C 8 days	Refrigerated 4°C 8 days
<i>Campylobacter jejuni</i> alone	Swab A	Swab B	Swab C
Seal scat spiked with <i>C. jejuni</i>	Swab D	Swab E	Swab F
Calf faeces spiked with <i>C. jejuni</i>	Swab G	Swab H	Swab I

- **Results and implications for processing of rectal swabs**

Campylobacter jejuni was isolated in pure culture on CCDA agar from the three untreated swabs (A, D and G) in the presence or absence of seal scat or calf faeces (Table 5-2). Culture obtained from the three frozen swabs (B, E and H) was comparable to that found in untreated swabs in all three treatment groups on CCDA agar. In refrigerated swabs (C, F and I), a light to moderate growth of suspected *Proteus* spp. was noted in both samples containing seal scat (F) or calf faeces (I).

Table 5-2 Results of freezing/refrigeration trial on growth of *Campylobacter* spiked faeces. Bacterial growth was recorded as 0 to +++. 0: No growth; +: light growth; ++: moderate growth; +++: heavy growth; *¹*C. jejuni* was recognised on CCDA plates as flat, medium sized, white/yellow colonies with a more pale yellow/white centre; *²*C. jejuni* colonies were recognised on Skirrow's agar as pinkish, shiny, discrete colonies; *³*Proteus* spp. was recognised as medium to large yellow to white, foul-smelling, spreading colonies on CCDA; *⁴*Proteus* spp. was recognised on Skirrow's agar as yellow-green, sticky, swarming colonies

Swab	Culture medium	No treatment	Frozen -80°C 8 days	Refrigerated 4°C 8 days
<i>Campylobacter jejuni</i> alone	CCDA	++ * ¹	++	+++
	Skirrow's agar		+++ * ²	++
Seal scat spiked with <i>C. jejuni</i>	CCDA	++	++	++ plus <i>Proteus</i> * ³ (++)
	Skirrow's agar		0 <i>Proteus</i> * ⁴ (++/+++)	0 <i>Proteus</i> (++/+++)
Calf faeces spiked with <i>C. jejuni</i>	CCDA	++	++	+/ <i>Proteus</i> (+/++)
	Skirrow's agar		++	+++ <i>Proteus</i> (++)

Although no replicate samples were used, results of this trial suggested that storage of faecal swabs spiked with *C. jejuni* at 4°C results in overgrowth of *Proteus* spp. bacteria when compared to rectal swabs stored at -80°C or processed immediately.

- **Limitations**

The faecal swabs were spiked with large numbers of *C. jejuni* which were likely to be substantially higher than that encountered in natural samples. Repeat sampling of this trial using replicate samples and serial dilutions of *C. jejuni* to assess sensitivity was not possible due to time constraints. Thus the effect of freezing rectal swabs on the chance of successfully isolating *Campylobacter* spp. was not reliably established.

- **Outcome**

Despite the logistical constraints involved with culture of *Campylobacter* spp., routine bacterial isolation methods for *Campylobacter* spp. (as described below), performed on the day of collection, were retained for this study rather than attempt to freeze rectal swabs thereby reducing the risk of false negative results.

5.2.2 Collection of samples

During autumn 2011, 90 wild-caught live grey seal pups from three distinct sites on the Isle of May, 19 live wild caught yearling grey seals on the Isle of May and 32 live grey seal pups that had stranded along the Scottish coastline were swabbed rectally for bacterial culture (see Chapter 2 for full details). Stranded seals were sampled with 24 hours of admission for rehabilitation to the Scottish SPCA National Wildlife Centre, in Fife, Scotland.

In addition, 50 dead free-ranging grey seal pups found on the Isle of May and 9 stranded grey seal pups that died or were euthanised on humane ground at the Scottish SPCA National

Wildlife Centre were swabbed rectally within 48h of death. A full post-mortem examination including extensive histopathology was performed on each of these 59 animals (see Chapter 3). Three sediment samples were also taken from each of two pupping locations within the colony (Tarbet slope and Rona Rocks).

Rectal swabs were placed in Amies transport medium with charcoal (Medical Wire and Equipment (MWE), Corsham, UK) and held at 4°C until processing which occurred within 8h in the field laboratory on the Isle of May for the free-ranging live pups, dead pups and live yearlings. Swabs from stranded live or dead grey seal pups were sent by first class post to SAC Consulting Veterinary Services, Inverness, where they were processed, incurring a delay of up to 4 days between sampling and processing in some cases.

5.2.3 Bacterial isolates and phenotypic characterisation

Rectal swabs from all wild-caught animals and sediment samples were plated onto CCDA (Oxoid) and incubated at 37°C under microaerophilic environment (Campygen sachets, Oxoid) in airtight containers and assessed at 48 hours, 4 days and 6 days post inoculation for suspect *Campylobacter* spp. colonies. These were recognisable as discrete irregularly-shaped to spreading, flat, colourless to pale white/grey colonies, often with a less dense central area. Raised, medium to large, discrete, opaque beige colonies (*Proteus* spp.) and tiny to medium sized, white, haemolytic bacterial colonies⁵ were deemed contaminants.

From each plate, a maximum of 3 distinct colonies were selected for further identification, sub-cultured onto Columbia agar with sheep blood plates (CSBA) (Oxoid) (Figure 5-1), incubated at 37°C under microaerophilic environment for 48h and stored at –80°C in Microbank bead vials (Pro-Lab Diagnostics, Neston, UK) until required.

⁵ Gram positive cocci, most likely *Streptococcus* spp.

Cultures were resuscitated from Microbank beads, inoculated onto CSBA (Oxoid), incubated at 37°C under microaerophilic conditions for 48h using the Oxoid Campygen system (Oxoid) and assessed for purity. Plates containing spreading colonies typical of *Campylobacter* (Figure 5-1) were selected for further examination.



Figure 5-1 *Campylobacter* spp. colonies resuscitated from a Microbank bead on Columbia agar with sheep blood (Oxoid). Colonies have a distinctive watery, milky-white, semi-translucent appearance and frequently coalesce.

Campylobacter identification was performed using Gram stain and a wide range of tests, largely described by On and Holmes (On et al. 1996; On & Holmes 1991b; On & Holmes 1992; On & Holmes 1995). Briefly biochemical assays tested the activity of catalase (Biomérieux, Basingstoke, UK), oxidase (1% w/v N-N'-N'-N'- tetramethyl-p-phenylenediamine dihydrochloride, Thermo Fisher Scientific, Loughborough, UK); hippuricase (hippurate

hydrolysis) (Rosco Diagnostica, Taastrup, Denmark); alkaline phosphatase (Rosco); urease (Rosco); gamma glutamyl aminopeptidase (Bioconnections, Leeds, UK); acetate esterase (Rosco) and the ability to reduce nitrate (Rosco). Sensitivity to nalidixic acid (Oxoid), cefoperazone (Oxoid), cephalexin (Oxoid) and bile (Rosco) along with H₂S production on Triple Sugar Iron (TSI) slopes (Oxoid) was assessed under a microaerophilic environment. Growth at 22°C, 25°C and 42°C on CSBA and growth on MacConkey agar (Oxoid), nutrient agar (Oxoid) supplemented with 1% glycine (Scientific Laboratory Supplies, Nottingham, UK), nutrient agar supplemented with 1.5% NaCl (Scientific Laboratory Supplies) and nutrient agar supplemented with 3.5% NaCl was assessed under a microaerophilic environment. Growth was assessed under anaerobic (Oxoid), CO₂ enriched (Oxoid) and aerobic atmospheres.

Following sequencing, due to a discrepancy in phenotype and sequence type, the hippurate hydrolysis test was repeated for all strains negative for hippurate hydrolysis (n=93) and three strains positive for hippurate hydrolysis (cultures 1, 2 and 5 - serving as positive controls for the hippurate hydrolysis test).

False negatives are found commonly with *C. jejuni*, on the hippurate hydrolysis test, either due to the isolate not expressing hippurate hydrolase despite possessing the gene, or due to operator error during execution of the test itself (Nakari et al. 2008; Rautelin et al. 1999; Totten et al. 1987). Bacteria such as *Campylobacter* spp. being highly fastidious in their growth requirements, frequently require increased concentrations of inoculum for successful outcomes for biochemical tests (Nakari et al. 2008). Therefore a much more dense bacterial suspension (McFarland standard 6) than that recommended by the manufacturer (McFarland standard 4) was prepared from the *Campylobacter* cultures and used for the biochemical tests. In addition, processing delay was kept to a minimum (<10 minutes) for this assay. A subset of these previously negative isolates was re-repeated to confirm the validity and specificity of the second series of results (6 isolates). Results were compared to the outcome of genotyping (considered

as the gold standard test) to assess sensitivity and specificity of this repeated test using the “epi.test” function of the “epiR” package.

5.2.4 Extraction and purification of genomic DNA

- **Strain selection**

For each individual animal from which *Campylobacter* was isolated, (*Campylobacter* positive animal), one to three strains were selected for whole genome sequencing on the basis of their phenotypic characteristics: arbitrarily hippurate hydrolysis⁶; reduction of nitrate; growth on agar supplemented with 1% glycine and resistance to nalidixic acid. Thus 90 strains of *Campylobacter* were selected from 74 seals and 2 sediment samples. Isolates were grown in a microaerophilic atmosphere at 37°C on CSBA plates (Oxoid) for 48h. Dense suspensions of bacterial culture were prepared in sterile PBS and 10µl of each suspension was plated onto a CSBA plate (Oxoid), incubated at 37°C under a microaerophilic environment for 48h to confirm the purity of the suspension.

- **Extraction**

Genomic DNA was isolated from 500µl of bacterial suspension, irrespective of optical density of the solution, using the Masterpure DNA purification kit (Epicentre, Madison, WI, USA), according to the manufacturer’s protocol with minor modifications (pellets were incubated at 65°C with proteinase K and tissue lysis buffer for 30 minutes; ribonuclease (RNase) incubation was extended to 2 hours and elution was carried out with 100µl elution buffer). DNA purity was assessed by measuring the optical absorbance values at wavelengths of 230, 260 and 280nm (A230, A260, and A280, respectively), using a NanoDrop 1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). A260/A280 and A260/A230 ratios were calculated

⁶ Initial set of hippurate hydrolysis test results

accordingly. Samples were quantitated using both the NanoDrop A260 absorbance and the PicoGreen dsDNA quantitation kit (Molecular Probes Inc., Invitrogen,). PicoGreen dsDNA quantitation was performed by ARK-Genomics (The Roslin Institute, Edinburgh, UK).

Quantitation using NanoDrop has the advantage of requiring only a very small sample volume (1µl) and indirectly infers DNA concentration by determining the absorbance of light at 260 nm (Gallagher & Desjardins 2006). The main disadvantage of this method is the contribution of signal from single-stranded DNA (ssDNA) and other contaminants, such as protein and extraction buffers (Gallagher & Desjardins 2006). PicoGreen reagent circumvents such contributions from interfering substances by exhibiting an emission maximum at 530nm when bound specifically to dsDNA (unbound PicoGreen reagent exhibits minimal fluorescence in solution) (Gallagher & Desjardins 2006). Values obtained with PicoGreen method were assumed to be of higher accuracy.

Following quantitation, based on results of the PicoGreen analysis, DNA samples were normalised to a concentration of 50ng/µl in 10mM Tris-HCl, pH 7.5. Nanodrop confirmed that the A260/A280 ratio was between 1.8 and 2 and that the A260/A230 ratio was between 2 and 2.2 for all samples. 4µl of diluted DNA was run on a 0.7% (w/v) agarose gel, prepared in 1 x Tris-acetate-EDTA (TAE) buffer supplemented with SafeView nucleic acid stain (NBS Biologicals Ltd, Huntingdon, UK), to assess DNA fragmentation and RNA contamination. The gel was visualised using a GelDoc system (Bio-Rad).

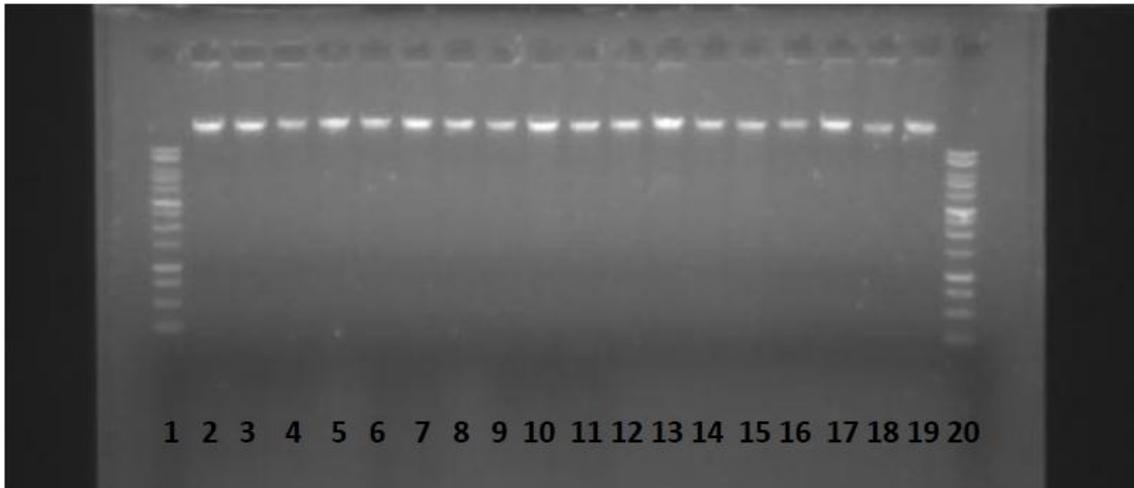


Figure 5-2 Gel image showing genomic DNA from 18 isolates of *Campylobacter* spp. (lanes 2-19). Each lane contains a single, non-smear band with no evidence of residual RNA. Lanes 1 and 20: 10kbp ladder.

5.2.5 Genome sequencing and assembly

Genome Sequencing was conducted at Glasgow Polyomics, Wolfson Wohl Cancer Research Centre, Glasgow, UK. Genomic DNA libraries were prepared using the Illumina TruSeq HT (High Throughput) DNA library preparation kit with dual indexing (Illumina, Saffron Walden, UK) following the manufacturers specifications with the following modifications: Samples were sheared on a Bioruptor Standard UCD 200TM sonicator (Diagenode S.A., Liège, Belgium) and were fragmented to approximately 300-500bp. For size selection, bands were cut from the gel at approximately 400-500bp to obtain an insert size of approximately 300-400 bp, accounting for the size of the adapters. Sequencing libraries were quantified using three methods: using the Qubit fluorometric assay (Life Technologies Ltd, Paisley, UK) as per manufacturer's instructions; using the Agilent 1000 kit (Agilent Technologies, Santa Clara, USA) and finally with the Kapa Library Quantification Kit - Illumina/Universal (quantitative PCR) (Kapa Biosystems, Woburn, MA, USA) as per manufacturer's instructions.

Sequencing was carried out using the Illumina Miseq (Illumina), as per manufacturer's instructions, with a 2x250bp dual index read (read length of 250bp and 8bp of index sequence).

Briefly, sequencing libraries were diluted to 4nM with elution buffer (10mM Tris pH8.5), denatured with 0.2M NaOH and then diluted to 12.5pM with HT1 hybridisation buffer (Illumina). Samples were combined and loaded onto the Flow cell at 12.5pM. Flowcell cluster amplification and sequencing were performed according to the manufacturer's protocols (Illumina) using MiSeq v1 cluster chemistry and flowcells. Read lengths were 2×250 bases. To assess quality of reads and overall coverage, forward and reverse reads were aligned to the reference genome sequence of *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 (ATCC 700819) using Bowtie2 software (ultrafast and memory-efficient tool for aligning sequencing reads to long reference sequences) (Langmead & Salzberg 2012).

De novo assemblies were performed using Velvet (Zerbino and Birney 2008) using a k-mer size of 49 and a coverage cut-off of 5. Following pan genome analysis, core genome analysis and MLST analysis of the assemblies obtained (see below), reference based assemblies were performed using Velvet with a k-mer size of 49 and a coverage cut-off of 5 using the genome sequence of *Campylobacter jejuni* subsp. *jejuni* M1 (NC_017280) for all isolates of *C. jejuni* within the clonal complex 45; using *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 (ATCC 700819) for all isolates of *C. coli* and all remaining isolates of *C. jejuni* and using the genome sequence of RM2100 *C. lari* (ATCC BAA-1060) for the two *C. lari* isolates. The best assembly per isolate was selected based on the lowest value of N50, which refers to a weighted median, with 50% of the entire assembly contained in contigs equal to or greater than this value.

5.2.6 Pan genome and core genome analysis

The accessory genome and binary presence/absence data, and core genome and single nucleotide polymorphisms (SNPs) of all 90 *Campylobacter* spp. isolates were extracted using the standalone-version of the Panseq program (Laing et al. 2010) and hierarchically clustered and visualized. Briefly, Panseq uses the MUMmer alignment algorithm (Kurtz et al. 2004) for

whole genome comparisons and the BLASTn algorithm for local sequence comparisons (Altschul et al. 1997) (<http://www.ncbi.nlm.nih.gov/BLAST/>). Analysis was carried out on all 90 genomes using a core genome threshold of 74; a percent identity cut-off of 90% and a fragmentation size of 500bp. The aligned fragments were visualized in SplitsTree 4 (<http://www.splitstree.org/>), using the uncorrected P distance and the neighbor-net algorithm (Huson & Bryant 2006).

Sequences were compared to known sequences of 16s rRNA using the NCBI BLAST database (<http://www.ncbi.nlm.nih.gov/BLAST/>). Megablast was carried out for 16s RNA sequences on both *C. lari* isolates to confirm that they had been assigned to the correct species (<http://www.ncbi.nlm.nih.gov/BLAST/>). In addition, short reads of both *C. lari* isolates were mapped onto a 1435bp partial sequence of 16S ribosomal RNA gene *Campylobacter lari* subsp. *lari* strain ATCC 35221 16S using the Burrows-Wheeler Aligner (bwa) algorithm (<http://bio-bwa.sourceforge.net/bwa.shtml>) (Li & Durbin 2009) and visualised using the Artemis visualisation platform (Carver et al. 2012).

5.2.7 Assignment of allele numbers, sequence types (ST) and phylogenetic analysis

- **MLST**

MLST genes alleles, STs and clonal complexes were determined for each isolate using the *Campylobacter* Multi Locus Sequence Typing website PubMLST (<http://pubmlst.org/Campylobacter/>) (Jolley & Maiden 2010). Seven MLST loci were used to type isolates of *C. coli* and *C. jejuni* which were internal fragments of the housekeeping genes encoding aspartase A (aspA), glutamine synthetase (glnA), citrate synthase (gltA), serine hydroxymethyltransferase (glyA), phosphoglucomutase (pgm), transketolase (tkt), and ATP synthase α subunit (uncA).

Seven MLST loci were used to also type isolates of *C. lari*. However these differed slightly as they were internal fragments of the genes encoding adenylate kinase (adk), ATP synthase alpha subunit (atpA), glnA, glyA, glucose-6-phosphate isomerase (pgi), pgm and tkt. Sequences of HipO (gene encoding hippurate hydrolase) were searched within the 90 genomes using the PubMLST website⁷. The presence or absence of this gene was recorded, along with the allele number (if present).

- **Phylogeny**

For initial phylogenetic analyses, START2 software was used to generate dendrograms from the concatenated MLST allele sequences of all *C. jejuni* and *C. coli* isolates using the unweighted pair group method with arithmetic mean (UPGMA) (Jolley et al. 2001).

For more detailed phylogenetic analyses, isolates of *C. jejuni* and *C. coli* from seal pups were compared with a broader collection of 192 previously published *C. jejuni* and *C. coli* genomes (Sheppard et al. 2013a; Sheppard et al. 2013b). This analysis was performed by G. Meric, Medical Microbiology and Infectious Diseases group, Swansea University, College of Medicine, Swansea, UK. Phylogenetic trees were constructed using concatenated sequences of 595 core genes shared by all *C. jejuni* and *C. coli*. Sequences were exported and aligned using built in functions in BIGSdb platform (Jolley & Maiden 2010). An approximation of the maximum likelihood algorithm was used to reconstruct the trees, using FastTree2 (Price et al. 2010). Trees were visualised from the Newick output of FastTree2 using MEGA2 (Kumar et al. 2001).

⁷ This publication made use of the *Campylobacter* Multi Locus Sequence Typing website (<http://pubmlst.org/Campylobacter/>) developed by Keith Jolley and sited at the University of Oxford (Jolley and Maiden 2010). The development of this site was funded by the Wellcome Trust.

- **Source attribution**

Source attribution was performed by S. Bayliss, Medical Microbiology and Infectious Diseases group, Swansea University, College of Medicine, Swansea, UK using the following methods: *C. jejuni* isolates from four putative source populations (chicken (n=1298), cattle (n=597), sheep (n=250) and wild bird (n=247) from previously published datasets (Sheppard et al. 2010a; Sheppard et al. 2011)) were compared to isolates of “unknown” origin (which in the present case corresponded to *C. jejuni* isolated from grey seals (n=74) or *Campylobacter* isolated from human clinical cases (n=1298), taken from the pubMLST database (<http://pubmlst.org/campylobacter/>) (Jolley and Maiden 2010)).

Prior to carrying out source attribution, the genetic heterogeneity within and between groups was analysed using ϕ -statistics using analyses of molecular variance in the ARLEQUIN 3.5 population genetics package (Excoffier & Lischer 2010). The seven MLST loci of each *C. jejuni* isolate were concatenated and the genetic distance between pairs of isolates was considered as the number of base pairs at which they differed. Sub-groupings within a population were defined as isolates derived from different publications. Permutation tests were used to assess significance, using 999 permutations. Genetic differentiation between groups, rather than within groups, and significance was performed using pairwise nested AMOVA. Low ϕ -statistic values indicated low genetic differentiation.

Empirical cross-validation was utilised to assess the robustness of the attribution model for application to the *C. jejuni* isolates used in the present study. This allowed for a number of parameters to be assessed; these parameters included the sensitivity of model to sample size variation, the suitability of the resulting approximations to be used for inference and the robustness of the approach to genetic heterogeneity. Empirical cross-validation was performed on 100 semi-randomised datasets which allowed for the adequate approximation of a number of

performance indicators. Each iteration of the empirical cross-validation was performed on a dataset of the non-human/non-seal samples in which approximately half of the samples were designated as test isolates. A total of 100 test isolate datasets underwent source attribution using standard attribution settings. From the resulting outputs three performance indicators were calculated: predicted proportion of isolates correctly assigned, bias, root mean square error (RMSE) and model coverage (the number of simulations out of 100 in which the 95% confidence interval incorporated the true value). These were calculated as previously detailed (Wilson et al. 2008).

Source attribution and associated analyses were performed using iSource (Wilson et al. 2008). The iSource software calculates source attribution probabilities by modelling the evolution of DNA sequences over time. The model incorporates parameters such as mutation and DNA exchange between populations, factors which would ultimately lead to differences in gene frequencies between populations. The model is a generalisation of Wright's island model called the migration matrix model (Kimura & Crow 1964). *Campylobacter* isolates from putative source populations (chicken, cattle, sheep and wild bird) were used to estimate migration, mutation and recombination. These estimates were used to generate posterior probability (F) and assign a source to a test isolate source. The relative contribution of each putative source population to the sequence of unknown origin (which in the present case corresponded to *Campylobacter* isolated from grey seals or *Campylobacter* isolated from human clinical cases) was calculated. Human and seal isolates were assumed to be representative of their source populations. The mean of the posterior distribution was used for point estimates and the 95% confidence intervals (CI) were calculated as 2.5% and 97.5% quantiles. The standard settings for the iSource software used during this study was 100,000 iterations. The state of the MCMC was recorded every 50 iterations and each run utilised a symmetrical Dirichlet prior with α of 1. For subsequent analysis a 1000 iteration burn-in was applied.

5.2.8 Immunohistochemistry

Immunohistochemistry was performed on selected sections of small and large intestine to detect *Campylobacter jejuni* (clone:Ab54125, Mouse monoclonal, Abcam Plc, Cambridge, UK, dilution 1:1000 in a 25% v/v solution of normal goat serum in PBS + 0.05% Tween₂₀) and visualised with an Envision™ kit (Dako, Ely, UK) as per manufacturer's instructions. Antigen retrieval involved microwave treatment at 121°C for 10 min, in citrate buffer pH 6.0. Endogenous peroxidase activity was blocked using a 3% solution of hydrogen peroxide in methanol (v/v) for 20mins at room temperature (RT). Nonspecific immunoglobulin binding was blocked by incubation of slides for 30 minutes with 25% v/v solution of normal goat serum in PBS Tween₂₀ (RT).

Caecum and colon of pigs experimentally infected with *Campylobacter jejuni* (MP09/1367, 1377) were used as positive control tissues. Negative-control sections were incubated with or isotype matched mouse IgM (dilution 1:500, Sigma M5909, 0.2 mg ml⁻¹) in place of the primary antibodies. The immunoreactions were visualised with Nova red (Vector Laboratories, Peterborough, UK; 10 mins at RT) and sections were counterstained with Mayer's haematoxylin.

5.2.9 Statistical analysis

Unless otherwise specified, all computations in this study were performed using the R statistical package (R Core Team 2013). For prevalence data, Fisher's exact test and Chi-squared tests were performed. Overall prevalence and odds ratios were calculated using a generalized linear model (GLM) with a binomial family and a logit link function. Site, sampling time, pup stage and the interactions between them were used as fixed explanatory factors. Statistical significance was set at $p \leq 0.05$.

5.3 Results

5.3.1 Prevalence

Campylobacter spp. were isolated from 75 of 181 (41.4%) grey seal pups and 2 of 6 sediment samples but were not isolated from any of the yearling samples (0/19). Between one and three isolates were cultured from each *Campylobacter* spp. positive animal resulting in 185 isolates available for further categorisation. The prevalence of *Campylobacter* in each respective group of seals is presented in Table 5-3.

Table 5-3 Prevalence of *Campylobacter* spp. isolated in different sample groups. Number of positive samples (% positive samples per group) [95% CI: 95% confidence interval]; (n=): number of pups/samples tested in each group

	All seals combined (n=194)	Yearlings	Pups				Sediment	
		Isle of May Live (n=19)	All pups (n=181)	Free-ranging		Stranded		Isle of May
				Dead (n=50)	Live (n=90)	Dead (n=9)	Live (n=32)	(n=6)
<i>Campylobacter</i> spp.	75 (37.5%)	0 (0%)	75 (41.4%)	24 (48%)	46 (51.1%)	1 (11.1%)	4 (12.5%)	2
95% CI	[31.1, 44.4%]	[0, 16.8%]	[34.5, 48.7%]	[34.8, 61.5%]	[41.0, 61.2%]	[5.7, 43.5%]	[5.0, 28.1%]	
<i>C. jejuni</i>	63 (31.5%)	0 (0%)	32 (17.7%)	18 (36%)	41 (45.6%)	1 (11.1%)	3 (9.4%)	2
96% CI	[25.5, 38.2%]	[0, 16.8%]	[12.8, 23.9%]	[24.1, 49.9%]	[35.7, 55.8%]	[0.6, 43.5%]	[3.2, 24.2%]	
<i>C. coli</i>	13 (6.5%)	0 (0%)	13 (7.2%)	6 (12%)	6 (6.6%)	0 (0%)	1 (3.1%)	0
96% CI	[3.8, 10.8%]	[0, 16.8%]	[0.4, 11.9%]	[5.6, 23.8%]	[3.1, 13.8%]	[0, 29.9%]	[0.2, 15.7%]	
<i>C. lari</i>	1 (0.5%)	0 (0%)	1 (0.5%)	0 (0%)	1 (1.1%)	0 (0%)	0 (0%)	1
96% CI	[0.02, 2.8%]	[0, 16.8%]	[0.02, 3.1%]	[0, 7.1%]	[0.05, 6.02%]	[0, 29.9%]	[0, 16.8%]	

The odds of harbouring *Campylobacter* spp. were 5.5 times higher (odds ratio = 5.55, $p < 0.001$) in free-ranging grey seal pups sampled on the Isle of May than in stranded grey seal pups sampled at the rehabilitation centre. No *Campylobacter* spp. bacteria were isolated from any of the live yearling grey seals on the Isle of May which was statistically significant when compared to live grey seal pups on the colony ($p < 0.001$). Among free-ranging pups, the prevalence of *Campylobacter* spp. was similar in both dead and live grey seal pups (48% and 51.1% respectively) and no significant differences were noted between free-ranging pups of different weight classes or pup stage (proxy for age).

Among free-ranging live grey seal pups, those sampled at the tidal boulder beach site had a 2 to 3 times lower odds of harbouring *Campylobacter* spp. when compared with seals sampled at the muddy, grassy slope site (OR=1.97; $p = 0.197$) or rocky pool site (OR=2.98; $p = 0.04$) (Table 5-4). Sampling time also influenced the likelihood of isolating *Campylobacter* spp. from live free-ranging grey seal pups with a statistically significantly lower prevalence in the mid season when compared to both early ($p = 0.021$) and late season ($p = 0.001$). Presence of *Campylobacter* spp. could not be correlated with pup stage (as a proxy for age). The final multivariate logistic regression model showed an association of *Campylobacter* spp. isolation with sampling time, as well as with pup stage (Table 5-5 and Figure 5-3). Seals sampled during the early or late pupping season were significantly less likely to harbour *Campylobacter* spp. when compared with those sampled in mid pupping season (OR=3.60, $p < 0.022$ and OR=6.42, $p < 0.001$, respectively). When controlling for sampling time, there was also an association between pup developmental stage and *Campylobacter* status, with stage II more likely to be positive than stage V pups (OR=0.43, 95% CI: 0.09-1.97, $p = 0.002$).

Table 5-4 Categorical risk factors, using univariate analysis, for all grey seals that are harbouring *Campylobacter* spp. (n=: group size; OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: p<0.05; **: p<0.01; ***: p<0.001)

	Risk factor	Group	(n=)	number (%) positive	OR	95% CI	P-value	Sign.
All seals	Group	Colony Live	90	46 (51.1%)	1	-	-	-
		Colony Dead	50	24 (48.0%)	0.88	0.44, 1.76	0.724	NS
		Stranded Live	31	4 (12.5%)	0.14	0.04, 0.42	<0.001	***
		Stranded dead	9	1 (11.1%)	0.12	0.01, 1	0.05	*
		Colony Live yearlings	19	0 (0%)	0	0, Inf	0.985	NS
All pups	Stranded/ Wild	Wild	140	70 (50.0%)	-	-	-	-
		Stranded	39	5 (12.5%)	0.14	0.05, 0.39	<0.001	***
Free ranging live seals only	Age	Pup	90	46 (51.1%)	1	-	-	-
		Yearling	19	0 (0%)	0	0, Inf	<0.001	***
Free ranging live and dead pups	Live/ dead	Dead	50	24 (48%)	1	-	-	-
		Live	90	46 (51.1%)	1.13	0.57, 2.26	0.724	NS
	Sex	Female	66	36 (54.5%)	1	-	-	-
		Male	73	33 (45.2%)	0.69	0.35, 1.34	0.272	NS
	Weight range (kg)	<19	14	7 (50%)	1	-	-	-
		19-25	73	34 (46.6%)	0.87	0.278, 2.74	0.81	NS
		25-31	13	6 (46.2%)	0.58	0.189, 3.89	0.84	NS
		>31	40	23 (57.5%)	1.35	0.40, 4.67	0.63	NS
	Pup stage	Stage 2	41	22 (53.7%)	1	-	-	-
		Stage 3	13	6 (46.2%)	0.74	0.21, 2.59	0.638	NS
Stage 4		27	15 (55.6%)	1.08	0.41, 2.87	0.878	NS	
Stage 5		9	3 (33.3%)	0.43	0.09, 1.97	0.278	NS	
Free ranging live pups only	Time point	Early	30	17 (56.7%)	3.6	1.22, 10.64	0.021	*
		Mid	30	8 (26.7%)	1	-	-	-
		Late	30	21 (70%)	6.42	2.08, 19.76	0.001	**
	Sampling site	Tidal boulder beach	30	11 (26.7%)	1	-	-	-
		Rocky pools	30	19 (63.3%)	2.98	1.04, 8.53	0.041	*
		Muddy grassy slope	30	16 (53.3%)	1.97	0.7, 5.54	0.197	NS

Table 5-5 Categorical risk factors, using multivariate generalised linear model analysis, for live, free-ranging grey seal pups only that are harbouring *Campylobacter* spp. on the Isle of May (95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: p<0.05; **: p<0.01; ***: p<0.001)

Risk factor	Group	Adjusted odds ratio	95% CI	P-value	Sign.
Sampling time	Early	3.6	1.22, 10.64	0.022	*
	Mid	1	-	-	-
	Late	6.42	2.08, 19.76	<0.001	***
Pup stage	Stage II	1	-	-	-
	Stage III	0.74	0.21, 2.59	0.345	NS
	Stage IV	1.08	0.41, 2.87	0.123	NS
	Stage V	0.43	0.09, 1.97	0.002	**

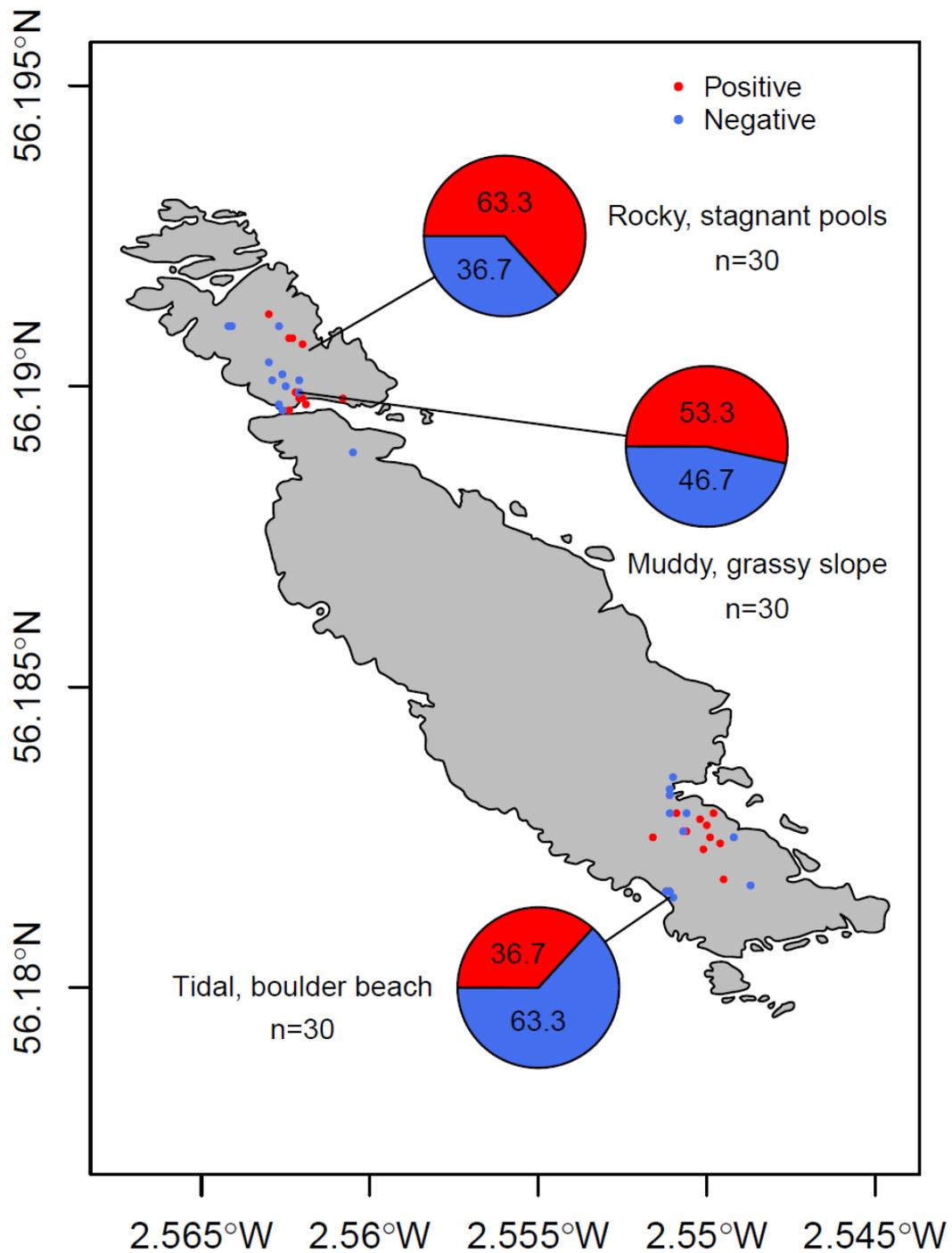


Figure 5-3 Summary map of the Isle of May breeding colony representing prevalence of *Campylobacter* spp. in grey seal pups. Prevalence in live pups is represented by pie charts with n=30 seal pups at each site (Tidal boulder beach, Muddy grassy slope and Rocky pools). Prevalence in dead pups is represented by scattered dots.

5.3.2 *Campylobacter* phenotypes

Following results of the genomic sequencing of the 90 selected isolates, 66.0% (31/47) of the sequenced strains which gave a negative result for hippurate hydrolysis were attributed to the *C. jejuni* species using MLST analysis. Furthermore, the hippurate gene was detected in all genome sequences of isolates attributed to *C. jejuni* on the basis of MLST. In light of this, the hippurate hydrolysis test was repeated for all strains for which the hippurate hydrolysis test had previously been negative, irrespective of *Campylobacter* species determined by MLST. In addition, as a positive control, the test was repeated on 3 strains previously positive for hippurate hydrolysis and known to be *C. jejuni* based on MLST attribution (Table 5-6).

Table 5-6 Results of second hippurate hydrolysis test performed on 50 isolates of *Campylobacter* of known species. The second test demonstrated very high sensitivity and specificity.

Species attributed using MLST	<i>C. jejuni</i>	<i>C. jejuni</i>	<i>C. coli</i>	<i>C.lari</i>
Outcome of initial hippurate assay result	Hippurate positive	Hippurate negative	Hippurate negative	Hippurate negative
Hippuricase (HipO) gene present	3/3	31/31	0/14	0/2
Outcome of second hippurate assay result	Hippurate Positive	3	31	0
	Hippurate Negative	0	0	14

Assuming MLST and the presence/absence of the hippuricase gene represents the gold standard, the sensitivity of the second test was 100% (95% CI: 85,100%) and the specificity was 100% (95% CI: 71,100%). Results of the second hippurate assay were retained on this basis.

Nine main phenotypical groups were identified based on the results of the oxidase test, growth at 42°C, growth in 1.5% NaCl, ability to hydrolyse hippurate*⁸ and resistance to nalidixic acid (Table 5-7).

5.3.3 Genomic DNA quality and quantitation

All samples of normalised genomic DNA gave distinct bands of regular intensity with no smearing or evidence of RNA contamination (Figure 5-2).

Nanodrop quantitation and picogreen quantitation prior to normalisation showed an approximately 2-3 fold over-estimation of DNA quantity using the Nanodrop when compared to Picrogreen assay.

5.3.4 Genomic assembly

The average number of contigs obtained using *de novo* genome assembly was 87.8 with an average N50 of 88,966. The average number of contigs obtained using reference based genome assembly was 73.2 with an average N50 of 134,340 (full details are provided in Appendix 2).

Preliminary MLST attribution of *de novo* assembled contigs using PubMLST suggested that of the 90 isolates: 74 were *C. jejuni*; 14 were *C. coli* and 2 isolates were most likely *C. lari*.

⁸ Results based on second Hippurate hydrolysis assay

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Table 5-7 Differential biochemical and phenotypical characteristics of 185 *Campylobacter* spp. isolates from grey seal pups and sediment. Nine distinct patterns were identified from this study (A – I); Characteristics of the related *Campylobacter* spp. are taken from works of (Foster et al. 2004; On et al. 1996; On 1996; Stoddard et al. 2007); Symbols: +, 90–100% of strains positive; (+), 80–89% of strains positive; d, 21–79% of strains positive; (-), 11–20% of strains positive; -, 0–10% of strains positive; w, weak. a: hippurate hydrolysis result of second test b: some strains positive/resistant; c : some strains negative/sensitive; NA : not available

Phenotype number or species	n	Presence of:		Hippurate hydrolysis ^a	Growth at/in:						Sensitivity to:		
		Catalase	Oxidase		25°C	42°C	1.5% NaCl	3.5% NaCl	1% Glycine	2% Bile	Nalidixic acid	Cephalothin	Cefoperazone
A	137	+	+	+	-	+	-	-	(+)	-	S	R	R
B	1	+	+	+	-	+	+	-	+	-	S	R	R
C	3	+	+	+	-	-	-	-	(d)	-	S	R	R
D	12	+	+	+	-	+	-	-	(d)	-	R	R	R
E	27	+	+	-	-	+	-	-	+	-	S	R	R
F	1	+	+	-	-	+	+	-	+	-	S	R	R
G	1	+	+	-	-	-	-	-	+	-	S	R	R
H	1	+	+	-	-	+	-	-	+	-	R	R	R
I	2	+	+	-	-	+	+	-	+	-	R	R	R
<i>C. coli</i>		+	+	-	-	+	-	-	+	(+)	S	R	R
<i>C. jejuni</i>		+	+	+	-	+	-	-	+	+	S	R	R
<i>C. lari</i>		+	+	-	-(b)	+	(+)	-	+	+	R	R	R(c)
<i>C. insulaenigrae</i>		+	+	-	-	+/-	-	-	+/-	NA	R	R/S	R
<i>C. upsaliensis</i>		-	+	-	-	+(c)	-	-	+	+	S	S	R

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Table 5-7 (cont.) Differential biochemical and phenotypical characteristics of 185 *Campylobacter* spp. isolates from grey seal pups and sediment. Nine distinct patterns were identified from this study (A – I); Characteristics of the related *Campylobacter* spp. are taken from works of Foster *et al.* 2004; On *et al.* 1996; On 1996 and Stoddard *et al.* 2007; Symbols: +, 90–100% of strains positive; (+), 80–89% of strains positive; d, 21–79% of strains positive; (-), 11–20% of strains positive; -, 0–10% of strains positive; w, weak; b: some strains positive/resistant; c : some strains negative/sensitive; NA : not available

Phenotype number or species	n	Growth at/in:				Presence of:				
		25°C	42°C	1.5% NaCl	3.5% NaCl	Urease	H ₂ S in TSI	ALP	GGA	Nitrate reductase
A	137	-	+	-	-	-	-	-	64 of 172	(+)
B	1	-	+	+	-	-	-	-	-	+
C	3	-	-	-	-	-	-	-	1 of 3	+
D	12	-	+	-	-	-	-	-	2 of 12	+
E	27	-	+	-	-	-	-	-	2 of 27	d
F	1	-	+	+	-	-	-	-	-	+
G	1	-	-	-	-	-	-	-	-	+
H	1	-	+	-	-	-	-	-	-	+
I	2	-	+	+	-	-	-	-	-	+
<i>C. coli</i>		-	+	-	-	-	(d)	-	NA	NA
<i>C. jejuni</i>		-	+	-	-	-	-	-	NA	NA
<i>C. lari</i>		-(b)	+	(+)	-	+/-	-	-(b)	NA	NA
<i>C. insulaenigrae</i>		-	+/-	-	-	-	-	NA	NA	NA
<i>C. upsaliensis</i>		-	+(c)	-	-	-	-	-	NA	NA

Analysis of the 16S rRNA gene sequences of both putative *C. lari* isolates using megablast (<http://www.ncbi.nlm.nih.gov/BLAST/>) indicated that both isolates were most closely related to the genome reference strains of *C. lari*, confirming their species identification. Sample JB109 showed over 99% homology with the 16S rRNA sequence of *C. lari* (RM2100 strain; ATCC BAA-1060D; NCBI reference sequence: NR_074555) with a discrepancy of only 3bp over a 1513 bp sequence; whereas sample JB188 showed 100% homology with the 1513 bp sequence.

Mapping of short reads from these two strains onto the 1513bp complete sequence of 16S rRNA gene from *C. lari* subsp. *lari* RM2100 strain (ATCC BAA-1060D) confirmed these findings. Three SNPs were detected after mapping of short reads from sample JB109 and no SNPs were identified for sample JB 188 (Figure 5-4).

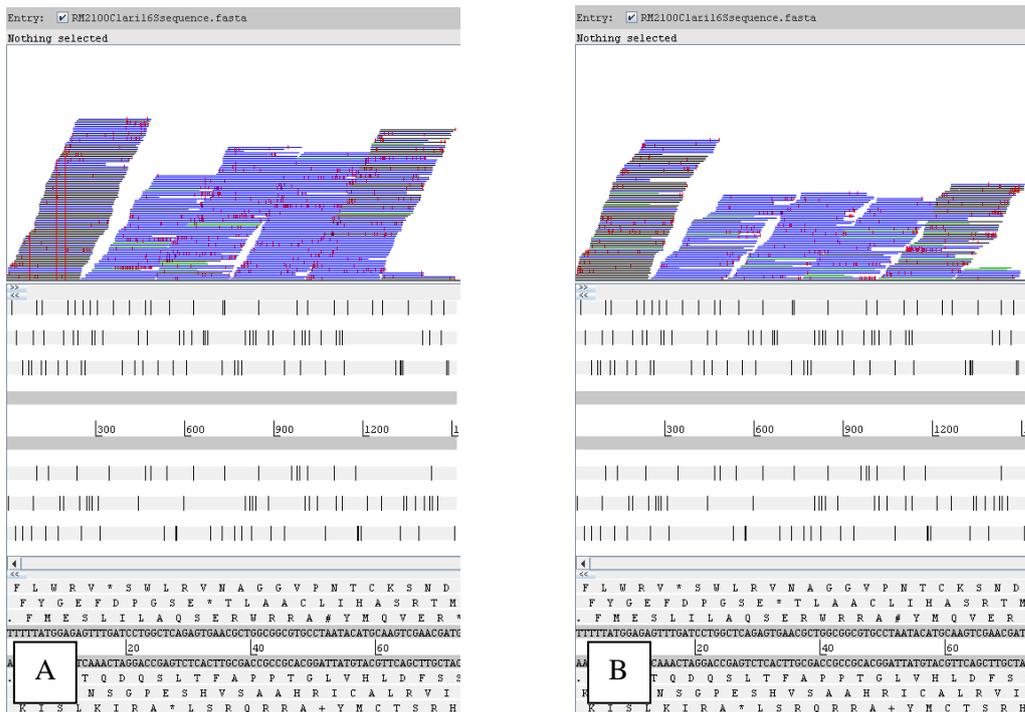


Figure 5-4 Short reads mapped directly onto complete sequence of 16S rRNA gene from *Campylobacter lari* subsp. *lari* RM2100 strain visualised using the Artemis genome browser. Vertical red lines on the reads indicate consistent differences to the reference sequence (SNPs). A: Isolate JB109: 3 SNPs appeared consistently in short reads (illustrated by red lines); B: Isolate JB188: No SNPs were identified

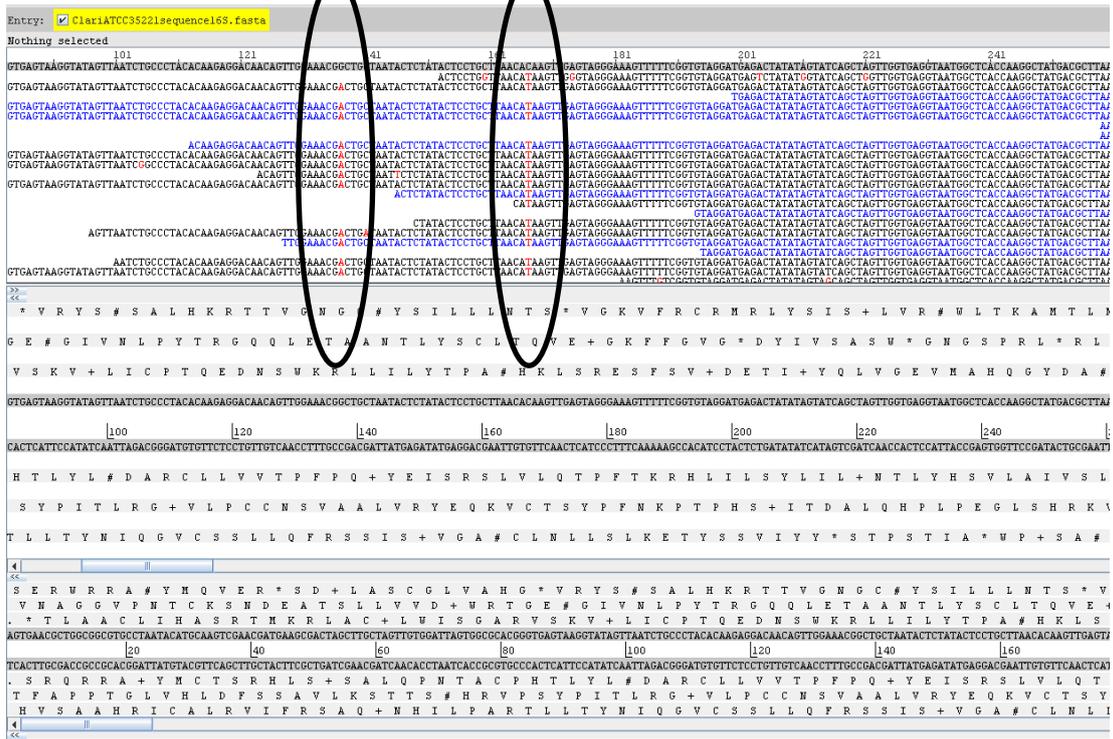


Figure 5-5 Short reads of isolate JB109 mapped onto a 1513bp sequence of 16S ribosomal RNA gene *Campylobacter lari* subsp. *lari* strain ATCC 35221 16S, partial sequence 16S (Accession number: NR_074555.1) visualised using the Artemis genome browser at the nucleotide level showing detail of 2 SNPs (red nucleotide indicates a difference from the reference sequence).

5.3.5 Sequence types of *Campylobacter* spp.

MLST was completed for each of the 90 isolates. Based on sequence types, 74 (82.2%) were confirmed as *C. jejuni*, 14 (15.6%) as *C. coli* and 2 (2.2%) as *C. lari*. The MLST assigned species differed from the original speciation test based on phenotypical characteristics alone with 31 (66%) of the hippurate negative isolates being reclassified as *C. jejuni*⁹. There were a total of 18 sequence types: 13 from *C. jejuni*, 3 from *C. coli* and 2 from *C. lari* (Table 5-9 and Figure 5-6).

For both isolates of *C. lari* only 6 and 5 MLST genes had corresponding alleles in the MLST database (samples 109 and 188, respectively).

⁹ Hippurate hydrolysis tests were repeated for these isolates as described in section 5.2.3

Table 5-8 Allele numbers for both isolates of *C. lari* identified in grey seals in this study

Isolate	Cla _{adk}	Cla _{atpA}	Cla _{glnA}	Cla _{glyA}	Cla _{pgi}	Cla _{pgm}	Cla _{tkt}
JB109	2	6	1	1	4	1	C ^{la} _{tktnovel1}
JB188	4	4	1	1	1	C ^{la} _{pgmnovel1}	C ^{la} _{tktnovel2}

Sequences of both isolates corresponding to MLST genes C^{la}_{pgm} and C^{la}_{tkt} were extracted using pubMLST and verified by re-mapping the Illumina short reads to the *C. lari* RM2100 reference genome. Three new alleles were thus determined and submitted to the pubMLST Non *jejuni/coli* *Campylobacter* database: C^{la}_{tktnovel1}, C^{la}_{tktnovel2} and C^{la}_{pgmnovel1}. Two new sequence types (ST) of *C. lari* were established. The three novel allele sequences are presented in Appendix 3.

Table 5-9 MLST profiles, sequence types and clonal complexes (CC) of the *C. jejuni* and *C. coli* isolates sequenced from grey seals. Freq.: Number of isolates of each sequence type. %: percentage of all strains sequenced. N.B.: Several identical STs were isolated from the same pup

Species	Clonal complex (CC)	Sequence type (ST)	MLST Profile							Freq.	%
			aspA	glnA	gltA	glyA	pgm	tkt	uncA		
<i>C. jejuni</i>	ST-45 CC	45	4	7	10	4	1	7	1	17	19.3
	ST-45 CC	583	4	7	10	4	42	51	1	13	14.8
	ST-45 CC	1326	104	7	10	4	1	7	1	2	2.3
	ST-45 CC	137	4	7	10	4	42	7	1	1	1.1
	ST-45 CC	1003	8	7	4	4	125	7	1	1	1.1
	ST-22 CC	22	1	3	6	4	3	3	3	14	15.9
	ST-21 CC	3853	2	1	21	334	2	1	5	9	10.2
	ST-21 CC	50	2	1	12	3	2	1	5	8	9.1
	ST-21 CC	21	2	1	1	3	2	1	5	5	5.7
	ST-1332 CC	696	2	1	4	28	58	25	58	1	1.1
	ST-1034 CC	4001	22	335	4	64	74	25	23	1	1.1
ND	1457	2	165	73	147	220	190	104	1	1.1	
ND	1256	10	8	34	6	39	88	3	1	1.1	
<i>C. coli</i>	ST-828 CC	827	33	39	30	82	104	56	17	12	13.6
	ST-828 CC	962	33	39	30	82	104	47	36	1	1.1
	ST-828 CC	1578	33	39	30	82	112	56	17	1	1.1

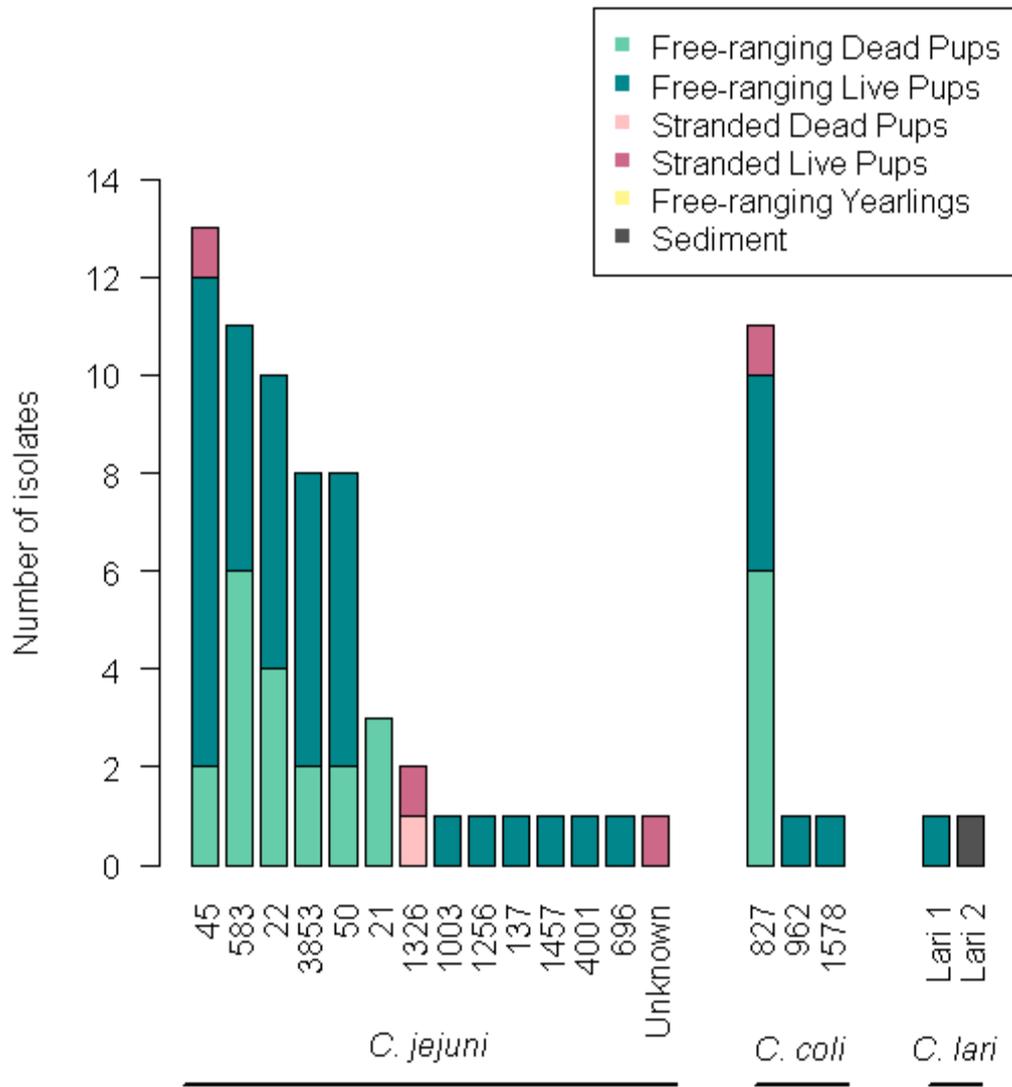


Figure 5-6 Number of grey seals positive for each sequence type of *Campylobacter* spp. per sample group after whole genome sequencing. Note: one pup (CD024) was positive for two STs of *C. jejuni* (ST-45 and ST-22) and is thus represented twice in this figure.

5.3.6 Phenotype, sequence type and gene expression

The hippurate hydrolysis gene (HipO) was present in all *C. jejuni* isolates and was absent in all *C. lari* and *C. coli* isolates. No correlation was found between the specific HipO allele and result of the initial hippurate hydrolysis assay. All 5 isolates of ST21, one isolate of ST22 and both

isolates of *C. lari* were resistant to nalidixic acid (Table 5-10). Both isolates of *C. lari* grew on agar supplemented with 1.5% NaCl.

Table 5-10 Phenotypical characteristics of each known sequence type of *Campylobacter* spp. isolated from grey seals. ST: sequence type; Nb: number of isolates of each known sequence type; 42°C: Growth at 42°C; Nalidixic acid: Resistance to Nalidixic acid; S: sensitive; R: Resistant; NaCl 1.5%: Growth in nutrient agar supplemented with 1.5% NaCl. *Several identical isolates may originate from a same pup. **Result of second hippurate hydrolysis assay.

Species	Clonal complex	ST	Nb*	Hippurate hydrolysis**	42°C	Nalidixic acid	NaCl 1.5%	
<i>C. jejuni</i>			21	5	+	2 of 5	R	-
	ST-21 Complex		50	8	+	6 of 8	S	-
			3853	9	+	+	S	-
	ST-1332 Complex		696	1	+	+	S	-
	Not defined		1457	1	+	+	S	-
	ST-1034 Complex		4001	1	+	+	S	-
	ST-22 Complex		22	14	+	+	13 of 14 S	-
			45	17	+	+	S	-
			137	1	+	+	S	-
	ST-45 Complex		1326	2	+	+	S	-
			583	13	+	12 of 13	S	-
			1003	1	+	+	S	-
	Not defined		1256	1	+	+	S	-
<i>C. coli</i>			827	12	-	+	S	-
	ST-828 Complex		1578	1	-	+	S	-
			962	1	-	+	S	-
<i>C. lari</i>	<i>C. lari</i>	<i>C. lari</i>	2	-	+	R	+	

5.3.7 Pan-genome analysis

Pan-genome analysis of all 90 *Campylobacter* spp. isolates sequenced revealed distinct clustering of isolates within bacterial species and within clonal complexes (Figure 5-7). This confirmed the attribution of MLST genes and STs and provided more subtle information on the relationship of each isolate within each clonal complex and sequence type (Figure 5-8).

The majority of isolates formed distinct clusters with very low genetic diversity between them. Within certain sequence types, there was evidence of more genetic diversity. In particular ST-45 contained a cluster of 14 apparently highly similar isolates, and 3 isolates separate from this cluster, demonstrating genetic divergence from this cluster (Figure 5-10).

Two of the four isolates found in stranded pups, (isolates 1 and 4) clustered tightly with isolates in ST-45 and ST-827, respectively.

5.3.8 Phylogenetic context of 72 *C. jejuni* genomes from seals

The *C. jejuni* isolates found in seals were widely spread throughout the known sequence types of this bacterial species. Strikingly, isolates from grey seals were consistently seen clustering with agricultural *Campylobacter*, indicating very recent divergences between them (Figure 5-11A).

Similar to that seen on the neighbour-net visualisation of the pan-genome (Figure 5-7 to 5-10), within each individual sequence type, the majority of isolates formed distinct clusters or “clones” with very low genetic diversity between them (Figure 5-11A). Again, there was evidence of some genetic diversity within each sequence type, in particular within ST-45 isolates (Figure 5-11A). Indeed, these three ST-45 isolates showed more similarity to isolates from cattle and wild birds than to the 14 other ST-45 isolates cultured from seals.

5.3.9 Phylogenetic context of 14 *C. coli* genomes from seals

Similar to that seen in the *C. jejuni* isolates, isolates originating from seals clustered with agricultural *Campylobacter* (Figure 5-11B). Of the 14 *C. coli* isolates sequenced, 12 belonged to ST-827 and formed a single, cluster or “clone”, similar to that described for the *C. jejuni* isolates. The two other *C. coli* isolates (ST-962 and ST-1578) were very closely related to the ST-827 isolates.

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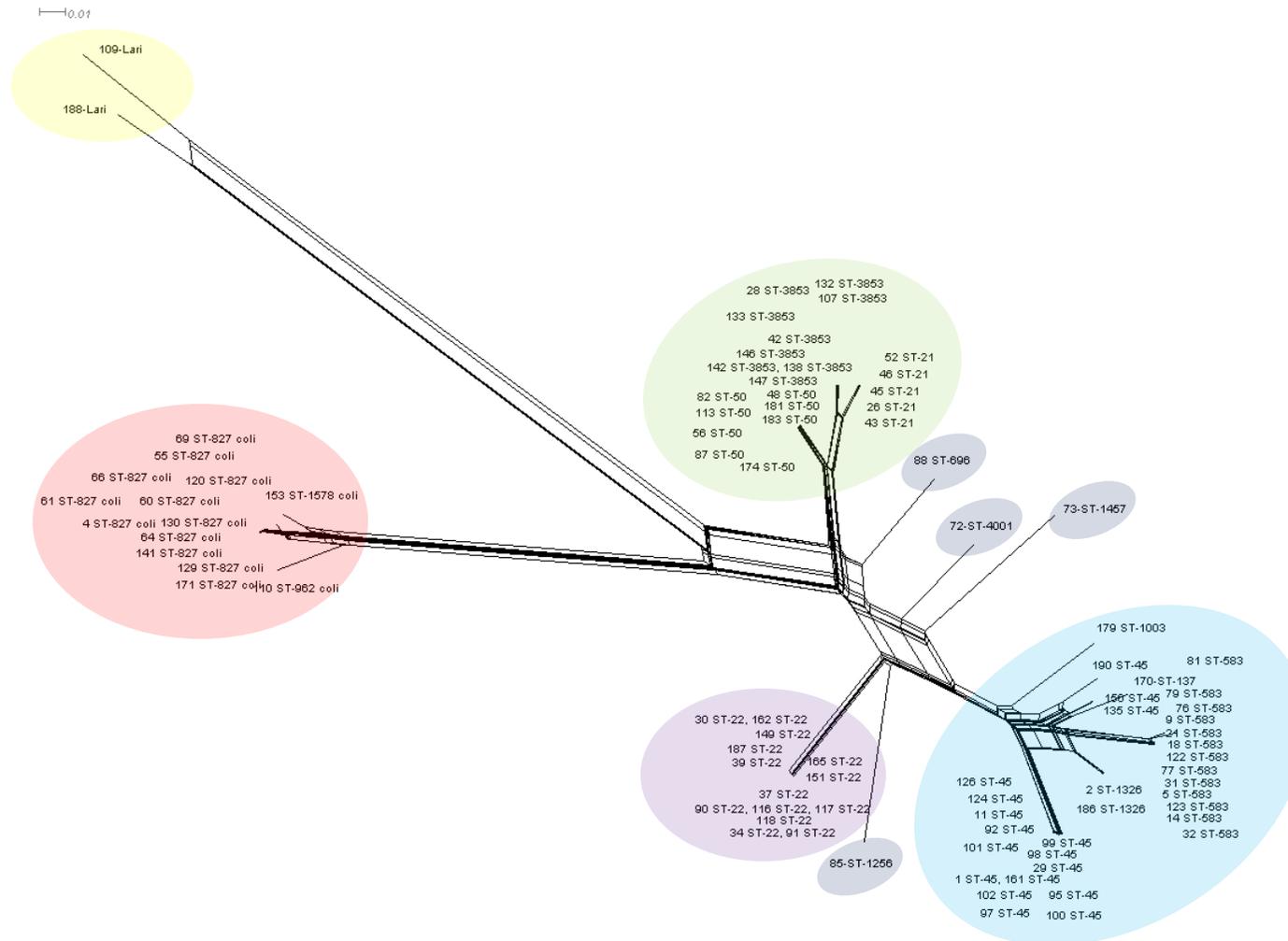


Figure 5-7 The neighbour-net visualization of the “pan-genome” among 90 *Campylobacter* spp. isolates sequenced from grey seals (Network generated from Panseq pangenome analysis and visualised in Splitstree). The “pan-genome” consists of 500 bp sequence fragments present in at least 74 bacterial genome sequences at a 90% sequence identity threshold. Labels represent the isolate number followed by the sequence type and/or species. Red shading: *C. coli*; Yellow shading: *C. lari*; Purple shading: *C. jejuni* Clonal complex (CC) 22; Blue shading: *C. jejuni* CC45; Green shading: *C. jejuni* CC50; Grey shading: *C. jejuni* of other CC.

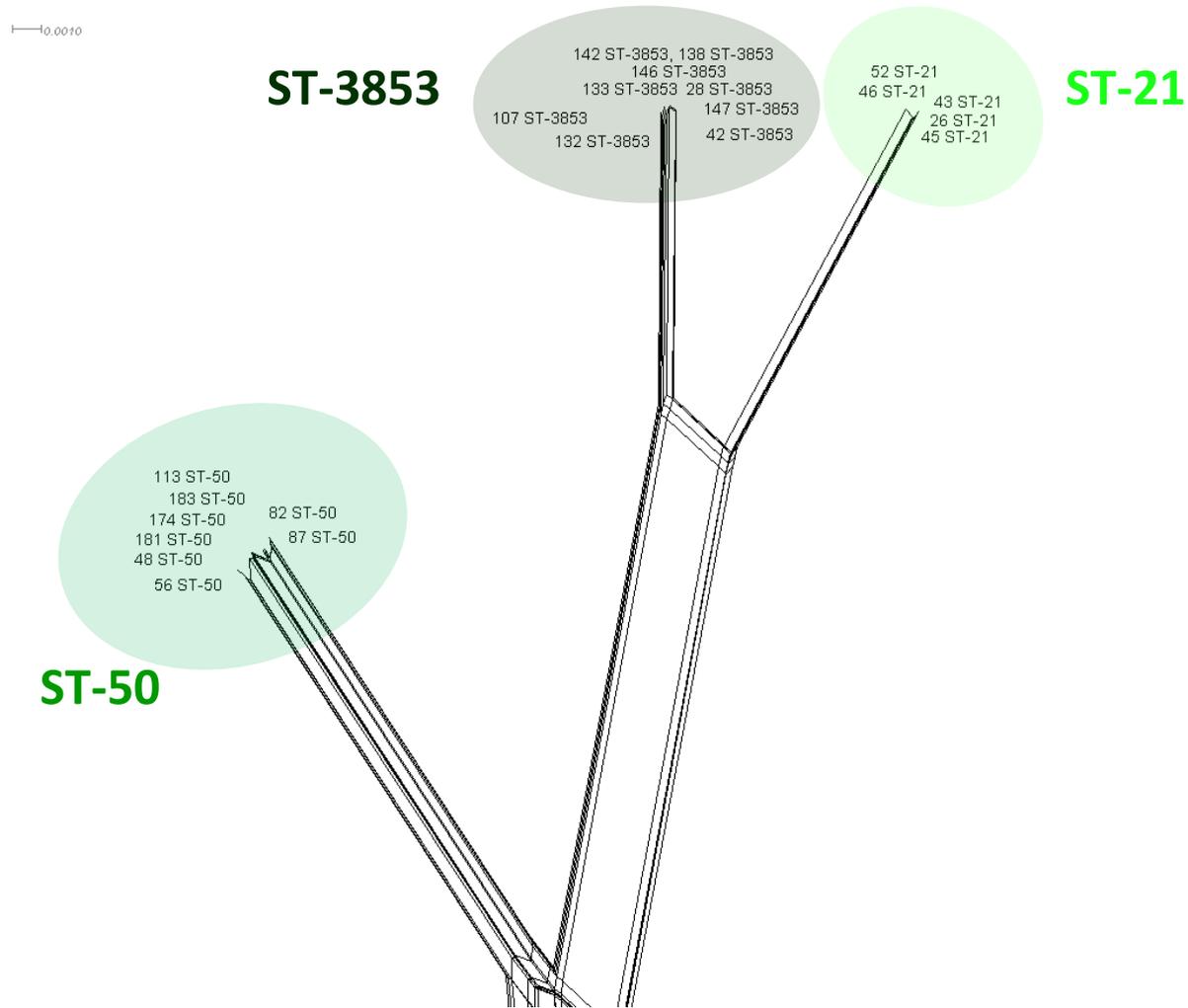


Figure 5-8 Detailed view of isolates within the ST-50 clonal complex of *C. jejuni*. Neighbour-net visualization of the “pan-genome” among 90 *Campylobacter* spp. isolates sequenced from grey seals (Network generated from Panseq pangene analysis and visualised in Splitsree). Labels represent the isolate number followed by the sequence type.

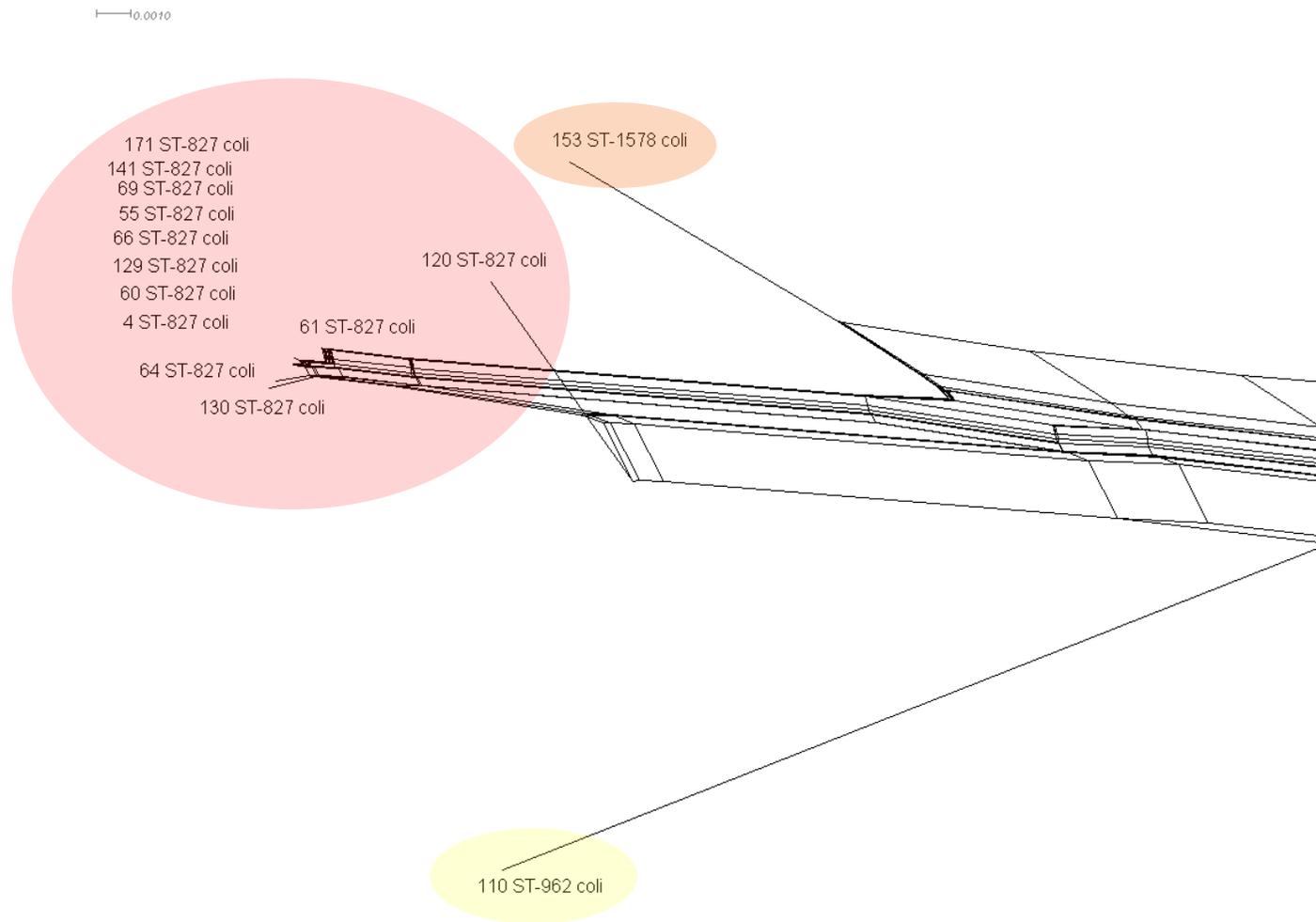


Figure 5-9 Detailed view of isolates within the ST-50 clonal complex of *C. jejuni*. Neighbour-net visualization of the “pan-genome” among 90 *Campylobacter* spp. isolates sequenced from grey seals (Network generated from Panseq pangenome analysis and visualised in Splitstree). Labels represent the isolate number followed by the sequence type. Shading represents individual sequence types.

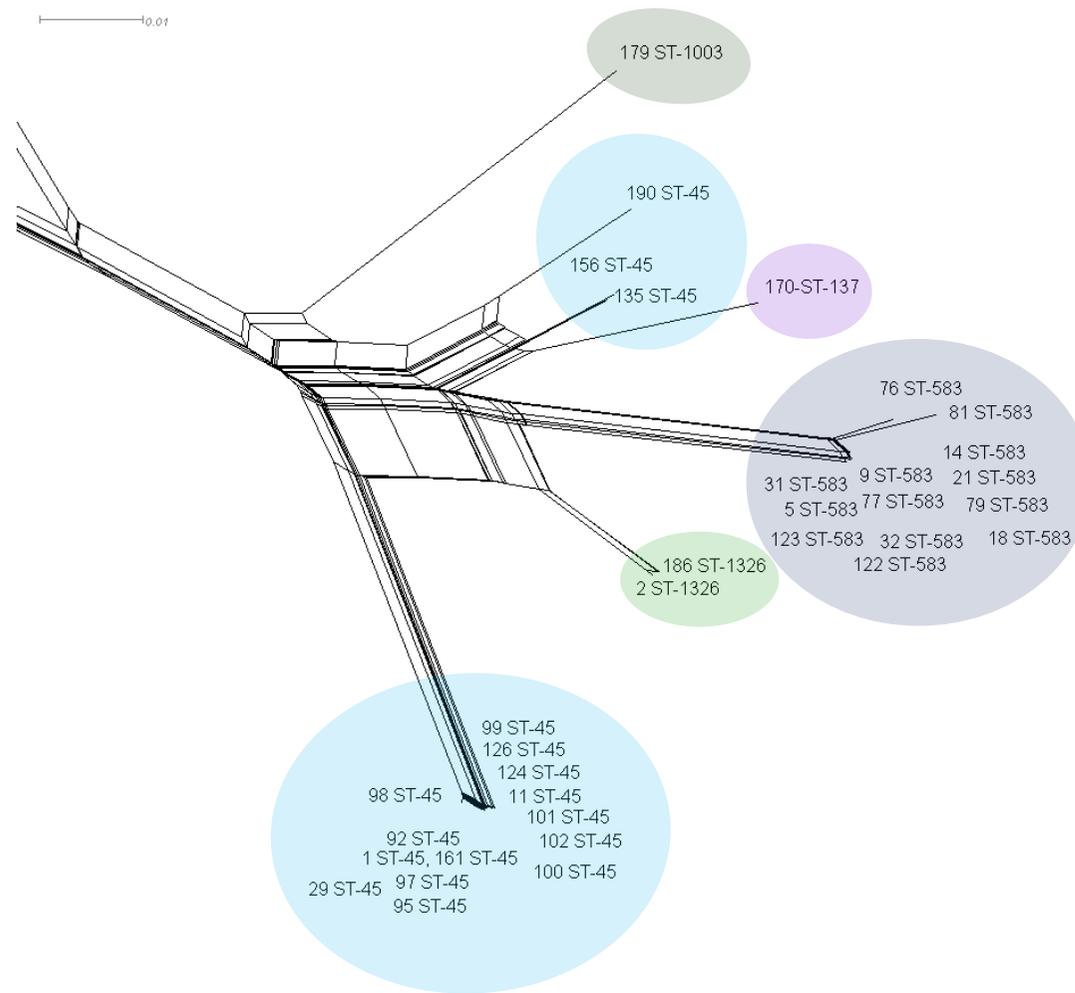


Figure 5-10 Detailed view of isolates within the ST-45 clonal complex of *C. jejuni*. Neighbour-net visualization of the “pan-genome” among 90 *Campylobacter* spp. isolates sequenced from grey seals (Network generated from Panseq pangenome analysis and visualised in Splitsree). Labels represent the isolate number followed by the sequence type. Shading represents individual sequence types. Note the two clusters of isolates corresponding to ST-45 in blue.

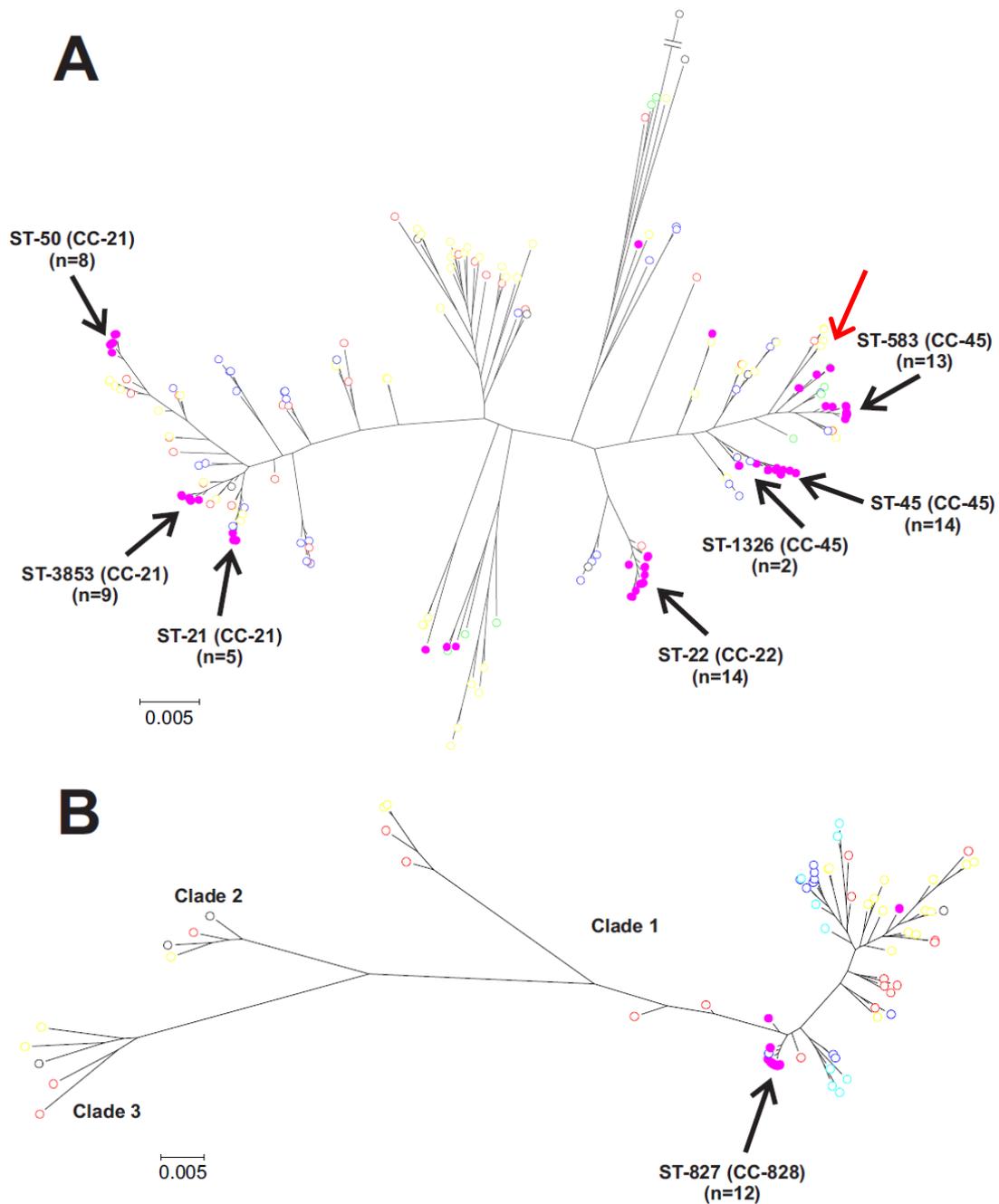


Figure 5-11 Phylogenetic tree of 74 *C. jejuni* (A) and 14 *C. coli* (B) isolates of grey seal origin compared to 192 *C. jejuni* and *C. coli* isolates of agricultural and non-agricultural sources based on concatenated sequences of 595 core genes established using an approximation of the maximum likelihood algorithm using FastTree2 and visualised using MEGA2. Pink circles represent isolates from grey seals (this study); yellow circles represent isolates from chicken; red circles represent isolates from humans; dark blue circles represent isolates from cattle; light blue circles represent isolates from sheep; green circles represent isolates from wild birds. The majority of isolates formed distinct clusters with very low genetic diversity between them or “epidemic clones” (black arrows). Isolates known to belong to ST-45 consisted of a principal “epidemic clone” containing 14 isolates and 3 distinct isolates which showed more genetic divergence (red arrow). Tree constructed by G. Meric, University of Swansea.

5.3.10 Source attribution of *C. jejuni* isolates

- **Diversity and differentiation of different *C. jejuni* populations**

As described by Wilson *et al.* (2008), assigning isolates to source populations with any degree of accuracy requires genetic differentiation between the groups over and above within-group heterogeneity. Table 5-11 presents the genetic differentiation within and between groups of the four putative source populations. There was significant heterogeneity within the groups that comprised more than one source type or study ($p < 0.05$). Genetic differentiation between subgroups ranged from 1.47% (chicken) to 10.6% (wild bird), suggesting that gene frequencies varied significantly between similar sources and between different studies of the same source. Genetic variation between each of the four groups was significant with ϕ_{GT} ranging from 1.01% (cattle vs sheep) to 18.44% (wild bird vs sheep).

Table 5-11 Genetic variation of *C. jejuni* within groups (ϕ_{ST}) and between groups (ϕ_{GT}). Significant ϕ -statistics are printed in bold. p: statistical p-value. Table constructed by S. Bayliss, University of Swansea.

Genetic differentiation within groups						
	Chicken	Cattle	Sheep	Wild Bird	Human	Seal
ϕ_{ST}	1.47 %	2.44 %	3.46 %	10.6 %	-	-
p	0.00	0.00	0.00	0.00		
Genetic variation between groups						
ϕ_{GT}/p	Chicken	Cattle	Sheep	Wild Bird	Human	Seal
Chicken	-	0.00	0.00	0.00	0.00	0.00
Cattle	7.85 %	-	0.00	0.00	0.00	0.00
Sheep	7.99 %	1.01 %	-	0.00	0.00	0.00
Wild Bird	12.36 %	16.77 %	18.44 %	-	0.00	0.00
Human	1.52 %	5.62 %	4.84 %	16.07 %	-	0.00
Seal	9.13 %	15.33 %	15.94 %	13.32 %	14.18 %	-

The significant variation of gene frequencies within groups could pose a potential concern for downstream analysis. Sub-grouping within population groups taken from multiple publications or locations could result in larger than expected linkage disequilibrium between loci and therefore skew the basis upon which source attribution was performed. Furthermore, although significantly different, the genetic variation between cattle and sheep groups remained small

(1.01%) and may have been masked by the larger genetic differentiation within groups (cattle 2.44%; sheep 3.46%). In light of this, empirical cross-validation was utilised to assess the robustness of the attribution model for application to the *C. jejuni* isolates used in the present study.

- **Empirical cross validation**

Empirical cross validation demonstrated that over 100 simulations 74% of test isolates were correctly assigned to their original source populations (Table 5-12). The model was considered to be well calibrated as it only slightly overestimated the true proportion (76% predicted to be correctly attributed). The overall proportion of cases attributable to chicken, sheep and wild-bird sources was slightly under-estimated. For sheep and wild birds, the model obtained the target coverage of 95 or above, whereas coverage was 89 and 84 for chicken and cattle, respectively, suggesting that there may be a small under-estimation of the proportion of cases attributable to these two sources.

Table 5-12 Performance of the source attribution model during empirical cross-validation. Isolates were assigned to a source population a posteriori based upon their most likely assignment probability (proportion of isolates correctly assigned). Coverage was the proportion of simulations where the true proportion fell within the 95% confidence interval. Bias and root mean square error (RMSE) were calculated for the sub-populations that were assigned to the pseudo-human group during empirical cross-validation. Table constructed by S. Bayliss, University of Swansea.

	Proportion of isolated assigned correctly		
	Predicted	Actual	
	0.76	0.74	
Population	Bias	RMSE	Coverage
Chicken	-0.02	0.03	89
Cattle	0.06	0.07	84
Sheep	-0.04	0.05	95
Wild Bird	-0.01	0.01	98

- **Source attribution**

The source attribution model described in the Materials and Methods was applied to the populations of human clinical *C. jejuni* isolates and *C. jejuni* isolates from grey seals,

separately. Isolates from both investigated populations were assigned to chicken, cattle, sheep and wild bird populations and the proportion of human and seal isolates attributable to each of the four source populations is presented in Figure 5-12.

The vast majority of human cases were attributable to livestock with only 1.43% (95% CI: 0.36, 3.25) of isolates attributed to wild birds. Chicken was the source of infection in the majority of cases (78.60%; 95% CI: 0.74-0.83), followed by sheep (10.51%; 95% CI: 2.26-20.31) and cattle (9.45%; 95% CI: 1.55-17.33). The 95% confidence intervals were wider for cattle and sheep than for other groups, reflecting the previously mentioned difficulty of differentiating these populations of *C. jejuni* from each other.

Similarly to that seen in human cases, chicken was found to represent the source of the grey seal isolates in the majority of cases (63.27%; 95% CI: 36.31-87.58), followed by sheep 18.30%; 95% CI: 1.04-44.36), wild bird (46%; 95% CI: 1.58-33.12) and cattle (4.97%; 95% CI 0.12-18.35). The 95% confidence intervals were wider than those seen in the human isolates, most likely due to the relatively small number of seal isolates examined.

The posterior probability of source assignment in the present study was assessed for each isolate, successively (Figure 5-13). Chicken was the dominant source in the vast majority of cases for both human and seal isolates. Approximately 25% of human and seal samples also had a strong probability of assignment to ruminant populations. Strikingly, the source probability of grey seal isolates mirrors that of *C. jejuni* isolates found in humans with the one clear difference between the human and seal populations being the increased probability of assignment to a wild bird source for almost every seal isolate.

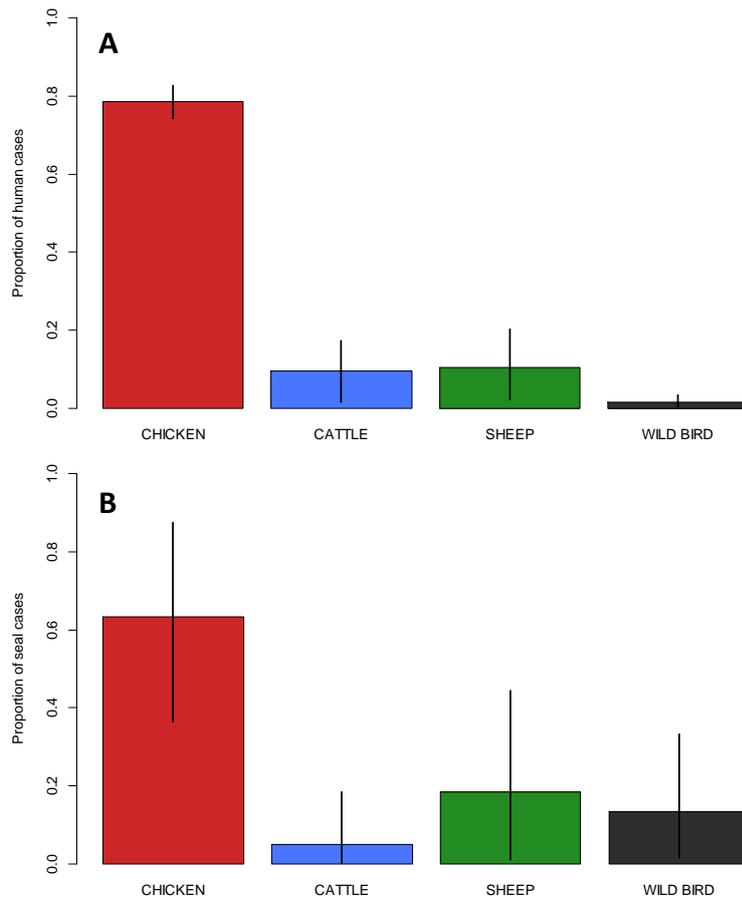


Figure 5-12 The estimated proportion of human (A) and seal (B) isolates of *Campylobacter jejuni* attributable to chicken, cattle, sheep and wild bird source populations. The 95% confidence intervals are indicated by a black line. Figure generated by S. Bayliss, University of Swansea using iSource software (Wilson et al. 2008)

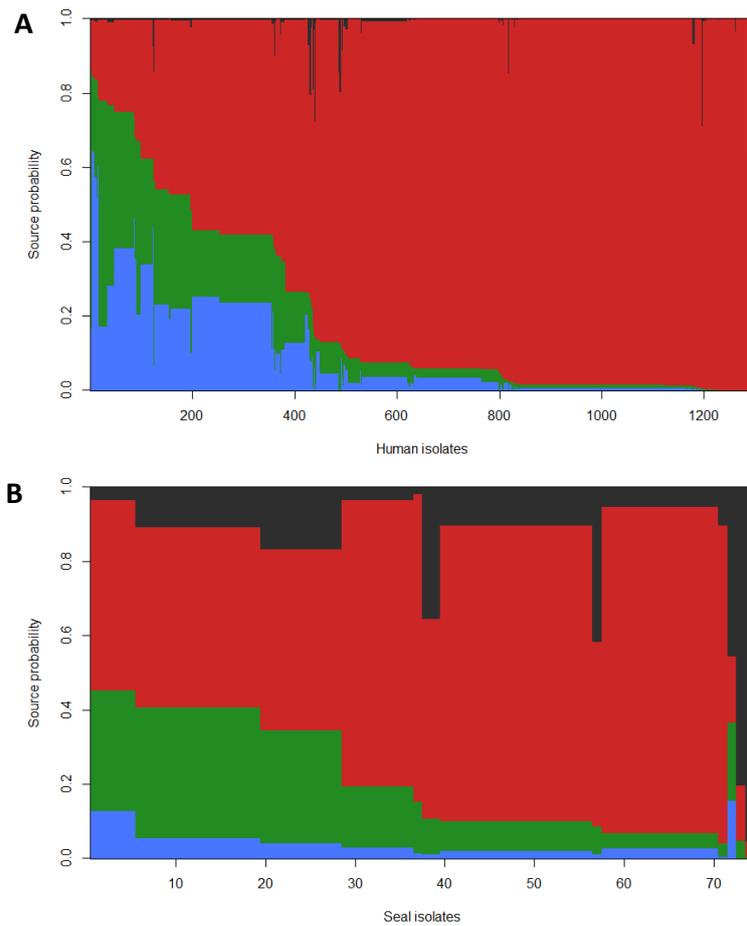


Figure 5-13 Probability of source assignment on an isolate by isolate basis for human (A) and seal (B) isolates of *Campylobacter jejuni*. The probabilities for each *C. jejuni* isolate are represented by a horizontal coloured bar for 1298 human isolates and 74 seal isolates. The colours represent chicken (red), cattle (blue), sheep (green) and wild bird (black). The isolates have been reordered to improve presentation. Figure generated by S. Bayliss, University of Swansea.

5.3.11 Epidemiology and distribution

In light of the unavoidable sampling bias introduced by the substantial delay in processing rectal swabs from stranded grey seal pups the epidemiology of *Campylobacter* spp. in free-living and stranded grey seals were investigated separately.

- **Free-living pups: Isle of May**

Odds ratio tables were established for the individual species *C. jejuni* and *C. coli* (see Table 5-13 and Table 5-14). Among live free-ranging grey seal pups, seal pups sampled at the tidal boulder beach site were 3.14 to 3.60 times less likely to harbour *C. jejuni* when compared with seals sampled at the muddy, grassy slope site (OR=3.14; $p<0.05$) or rocky pool site (OR=3.6; $p<0.05$). Sampling time also influenced the likelihood of isolating *C. jejuni* from live free-ranging grey seal pups with a statistically significantly lower prevalence in the mid season when compared to both early ($p=0.005$) and late season ($p=0.002$).

No risk factors were determined for *C. coli* carriage (Table 5-14).

The distribution of sequence types and clonal complexes of *Campylobacter* spp. within the Isle of May was geographically widespread. The majority of clonal complexes were present in all locations sampled with the exception that isolates belonging to the ST-22 clonal complex were not found in the southern half of the Isle of May in either dead or live grey seal pups. In addition, only single isolates of *Campylobacter* spp. were recorded from CC-ST-1034; CC-ST-1332 and from two undefined clonal complexes (ST-1256 and ST-1457) making it difficult to comment on spatial distribution.

Table 5-13 Categorical risk factors, using univariate analysis, for grey seals that are harbouring *Campylobacter jejuni*. (n=: group size; OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: p<0.05; **: p<0.01; ***: p<0.001)

	Risk factor	Group	(n=)	number (%) positive	OR	95% CI	P-value	Sign.
All seals	Group	Colony Live	90	41 (45.5%)	1.00			
		Colony Dead	50	18 (36%)	0.67	0.33, 1.37	0.274	NS
		Stranded Live	31	2 (6.5%)	0.08	0.02, 0.37	0.001	**
		Stranded dead	9	1 (11.1%)	0.15	0.02, 1.24	0.079	NS
		Colony Live yearlings	19	0 (0%)	0.00	0, Inf	0.985	NS
All pups	Stranded/ Wild	Wild	140	59 (42.1%)	1.00			
		Stranded	39	3 (7.7%)	0.11	0.03, 0.39	<0.001	***
Free ranging live seals only	Age	Pup	90	41 (54.4%)	1.00			
		Yearling	19	0 (0%)	0.00	0, Inf	<0.001	***
Free ranging live and dead pups	Live/ Dead	Dead	50	18 (36%)	1.00			
		Live	90	41 (45.6%)	1.49	0.73, 3.03	0.271	NS
	Sex	Female	66	29 (43.9%)	1			
		Male	73	29 (39.7%)	0.84	0.43, 1.65	0.615	NS
	Weight range (kg)	<12	14	5 (35.7%)	1			
		12-25	73	28 (38.4%)	1.12	0.34, 3.68	0.85	NS
		25-31	13	5 (38.5%)	1.13	0.24, 5.37	0.88	NS
		>31	40	21 (52.5%)	1.99	0.56, 6.99	0.28	NS
	Pup stage	Stage 2	41	19 (46.3%)	1.00			
		Stage 3	13	5 (38.5%)	0.72	0, 2.59	0.619	NS
Stage 4		27	15 (55.6%)	1.45	0.55, 3.84	0.458	NS	
Stage 5		9	2 (22.2%)	0.33	0.06, 1.79	0.199	NS	
Free ranging live pups only	Time point	Early	30	17 (56.7%)	5.23	1.66, 16.51	0.005	**
		Mid	30	6 (20%)	1.00			
		Late	30	18 (60%)	6.00	1.89, 19.04	0.002	**
	Sampling site	Tidal boulder beach	30	8 (26.7%)	1.00			
		Rocky pools	30	17 (56.7%)	3.60	1.22, 10.64	0.021	*
		Muddy grassy slope	30	16 (53.3%)	3.14	1.07, 9.27	0.037	*

Table 5-14 Categorical risk factors, using univariate analysis, for grey seals that are harbouring *Campylobacter coli*. (n=: group size; OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: p<0.05; **: p<0.01; ***: p<0.001)

	Risk factor	Group	(n=)	number (%) positive	OR	95% CI	P-value	Sign.
All seals	Group	Colony Live	90	6 (6.7%)	1.00	-	-	-
		Colony Dead	50	6 (12%)	1.91	0.58, 6.27	0.29	NS
		Stranded Live	31	1 (3.2%)	0.47	0.05, 4.04	0.49	NS
		Stranded dead	9	0 (0%)	0	0, Inf	0.99	NS
		Colony Live yearlings	19	0 (0%)	0	0, Inf	0.99	NS
All pups	Stranded/Wild	Wild	140	12 (8.6%)	1.00	-	-	-
		Stranded	39	1 (2.6%)	0.28	0.04, 2.23	0.229	NS
Free ranging live seals only	Age	Pup	90	6 (6.7%)	1.00	-	-	-
		Yearling	19	0 (0%)	0.00	0, Inf	<0.001	***
Free ranging live and dead pups	Live/dead	Dead	50	6 (12%)	1.00	-	-	-
		Live	90	6 (6.7%)	0.52	0.16, 1.72	0.286	NS
	Sex	Female	66	7 (10.6%)	1	-	-	-
		Male	73	5 (6.8%)	0.62	0.19, 2.06	0.434	NS
	Weight range (kg)	<19	14	2 (14.3%)	1	-	-	-
		19-25	73	5 (6.8%)	0.44	0.08, 2.54	0.36	NS
		25-31	13	2 (15.5%)	1.09	0.13, 9.12	0.94	NS
		>31	40	3 (7.5%)	0.49	0.07, 3.27	0.46	NS
	Pup stage	Stage 2	41	2 (4.9%)	1.00	-	-	-
		Stage 3	13	1 (7.7%)	1.62	0.14, 19.52	0.702	NS
Stage 4		27	2 (7.4%)	1.56	0.21, 11.8	0.667	NS	
Stage 5		9	1 (11.1%)	2.44	0.2, 30.24	0.488	NS	
Free ranging live pups only	Time point	Early	30	0 (0%)	0.00	0, Inf	0.993	NS
		Mid	30	2 (6.7%)	1.00	-	-	-
		Late	30	4 (13.3%)	2.15	0.36, 12.76	0.398	NS
	Sampling site	Tidal boulder beach	30	2 (6.7%)	1.00	-	-	-
		Rocky pools	30	3 (10%)	1.56	0.24, 10.05	0.643	NS
		Muddy grassy slope	30	1 (3.33%)	0.48	0.04, 5.63	0.561	NS

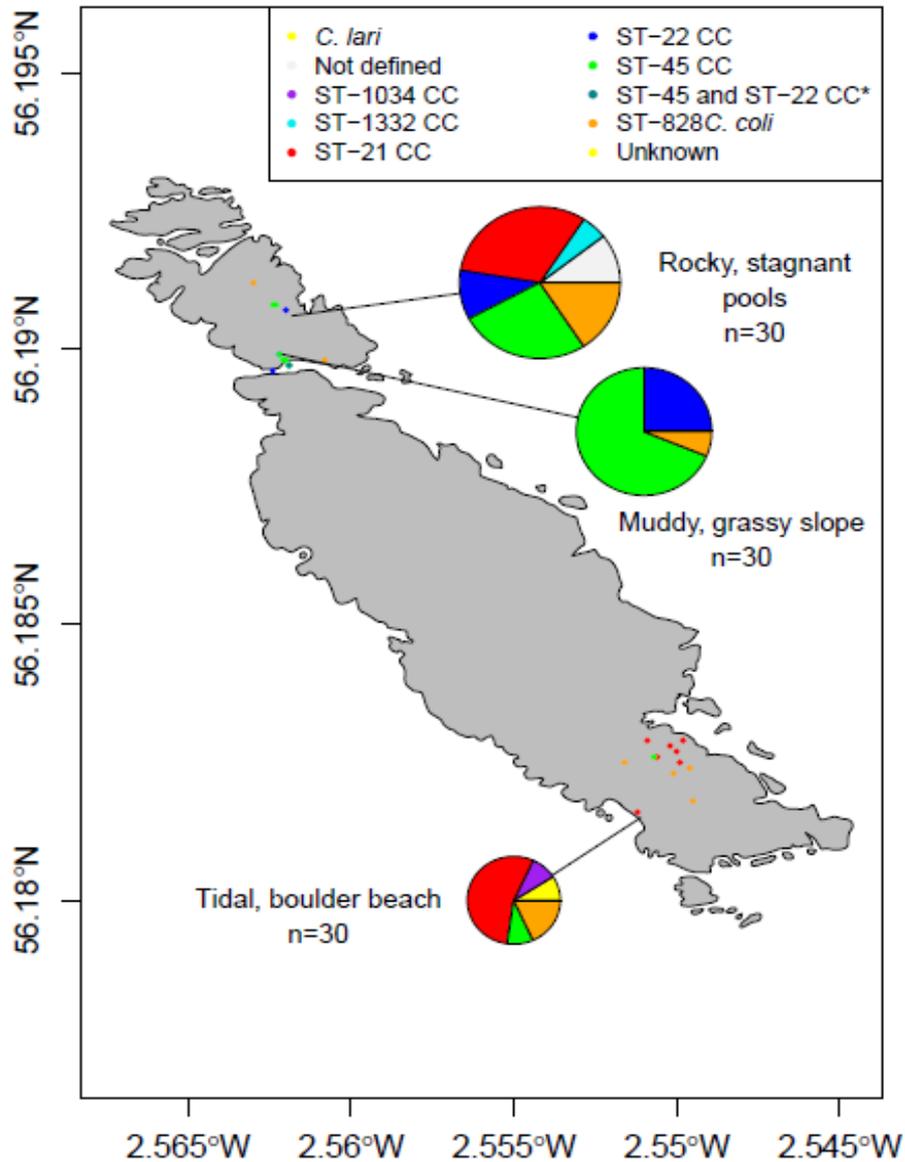


Figure 5-14 Distribution of *Campylobacter* spp. strains on the Isle of May grey seal breeding colony by clonal complex or species. Distribution in live pups is represented by pie charts with n=30 seal pups at each site (Tidal boulder beach, Muddy, grassy slope and Rocky pools) and size of the pie chart is proportional to the number of positive animals at each of the three sites. Distribution and clonal complexes of *Campylobacter* spp. in dead pups is represented by coloured dots. * One dead pup harboured two *C. jejuni* belonging to two clonal complexes (C-45 and CC-22)

- **Stranded grey seal pups**

Four of 32 (12.5%) grey seal pups had a positive culture of *Campylobacter* spp. from rectal swabs on arrival at the rehabilitation centre. The stranding location of these four pups and sequence types isolated were diverse (Figure 5-15). A *Campylobacter* sp. was isolated from only one dead, stranded grey seal pup (R006) which had been negative for *Campylobacter* spp. on arrival (previous reference: A001). This pup carried ST 1326 (CC ST-45), the same sequence type as that found in pup A007, present at the rehabilitation centre at the same time as R006. Both pups were found stranded in Caithness.

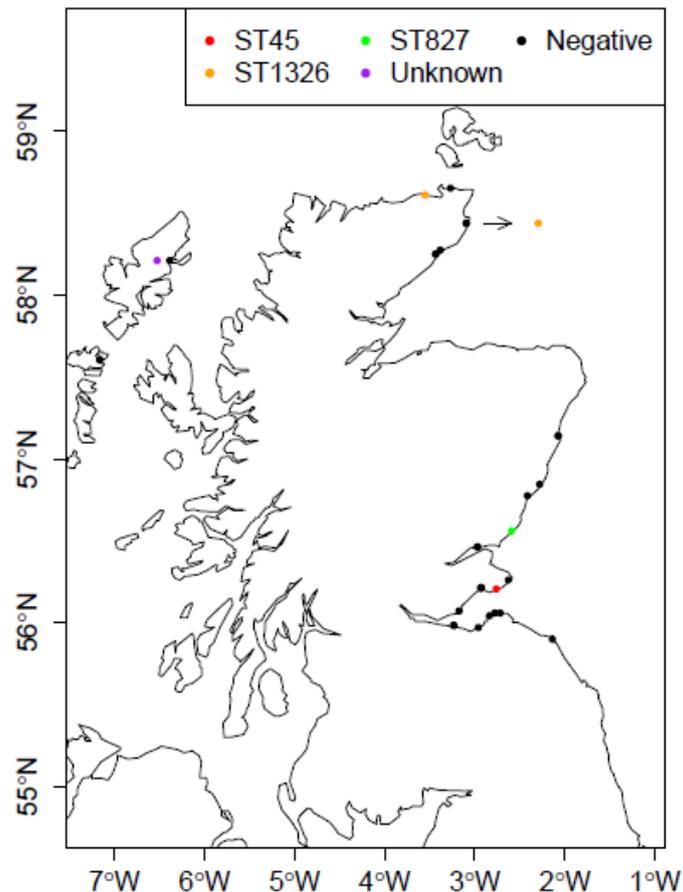


Figure 5-15 Map of Scotland showing the stranding locations of all live grey pups sampled for *Campylobacter* spp. on arrival at the Scottish SPCA National Wildlife Centre. Colour of dot represents the sequence type of *Campylobacter* spp. (if known). Arrow and orange dot represent isolation of ST1326 from a stranded pup, negative on arrival but found positive at the time of post-mortem examination.

5.3.12 Pathogenicity

- **Histopathology**

Histological examination of sections of large and small intestine showed a statistically significant correlation between isolation of *Campylobacter* spp. from rectal swabs and the presence of moderate to severe colitis (Fisher's exact test, $p=0.02$) in dead grey seal pups on the Isle of May. In addition, the combination of crypt abscessation in the large intestine and/or presence of mucosal inflammation, irrespective of severity, also correlated with the presence of *Campylobacter* spp. in rectal swabs ($p=0.0404$).

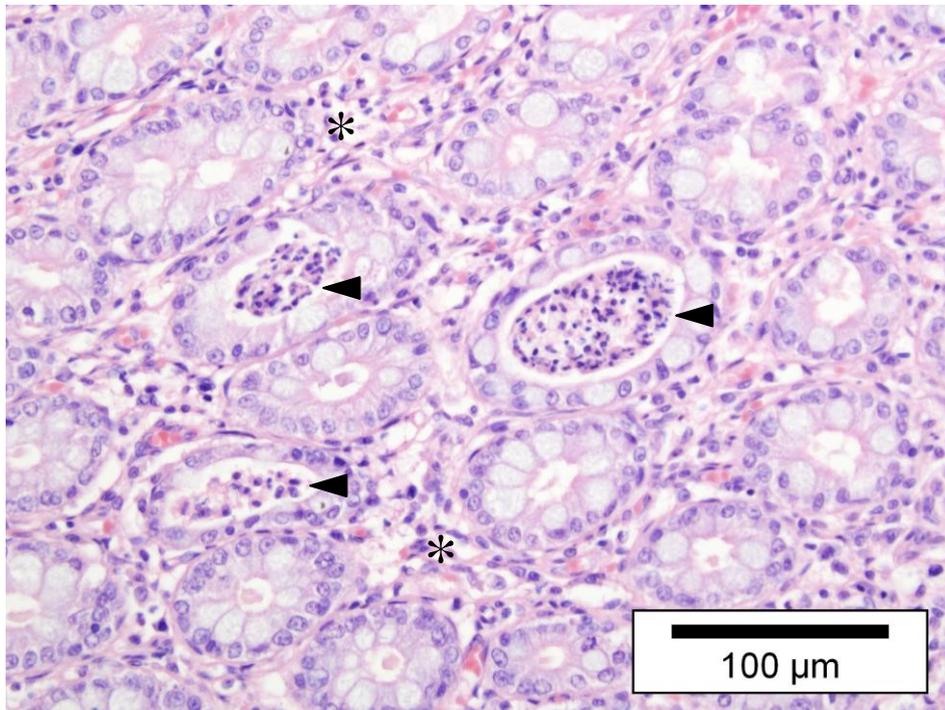


Figure 5-16 Large intestine. Crypt abscesses (arrowheads) containing degenerate neutrophils with moderate, diffuse suppurative enteritis expanding the lamina propria (*) (Animal ID: CD038). H&E. x200.

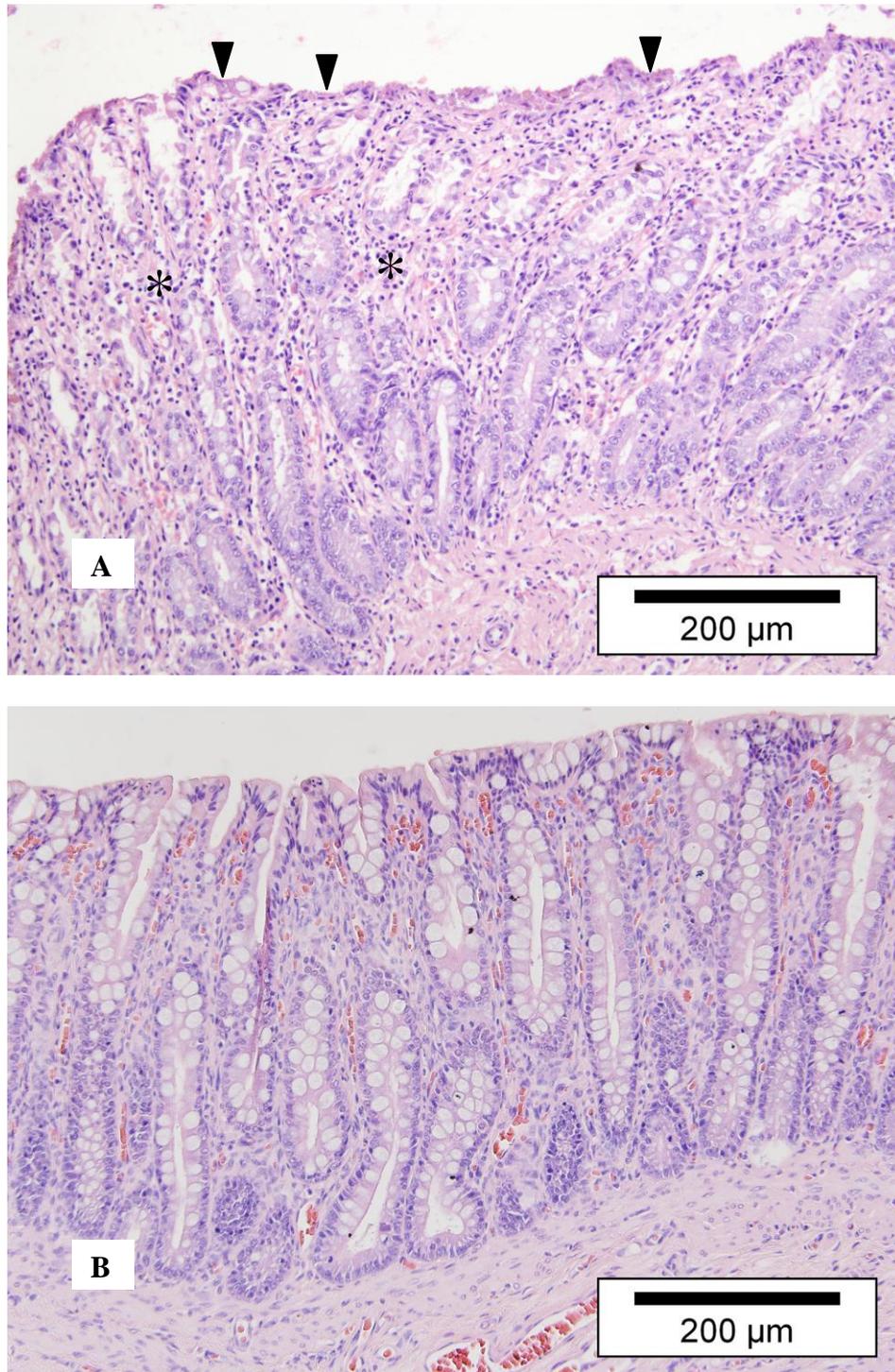


Figure 5-17 A. Colon of pup CD038 showing suppurative colitis (inflammation of the lamina propria (*)) and erosion of the overlying enterocytes (arrowheads). B: Colon of pup CD015. Normal morphology for comparison. H&E. x100.

- **Immunohistochemistry**

Campylobacter spp. organisms were located within sections of small and large intestine using specific immunohistochemistry. Negative control preparations showed only a small amount of ill-defined labelling in the lumen of the small and large intestine which was associated with large aggregates of digesta. Well defined curved bacterial organisms, consistent with *Campylobacter* spp., were labelled within intestinal crypts (Figure 5-18). Bacteria were located on the surface of enterocytes but did not show any evidence of invasion into enterocytes (Figure 5-19). No immunolabelling was noted in the tissues of either of the two stillborn pups, from which no *Campylobacter* spp. were isolated.

- **Co-infection with *Salmonella* spp.**

A linear regression model investigating whether moderate to severe colitis was correlated with the presence of *Campylobacter*, *Salmonella* or the interaction of these two factors showed that neither *Salmonella* nor the interaction of these two terms were significant. Therefore there was no evidence that colitis is exacerbated by the presence of *Salmonella* when controlling for *Campylobacter* infection.

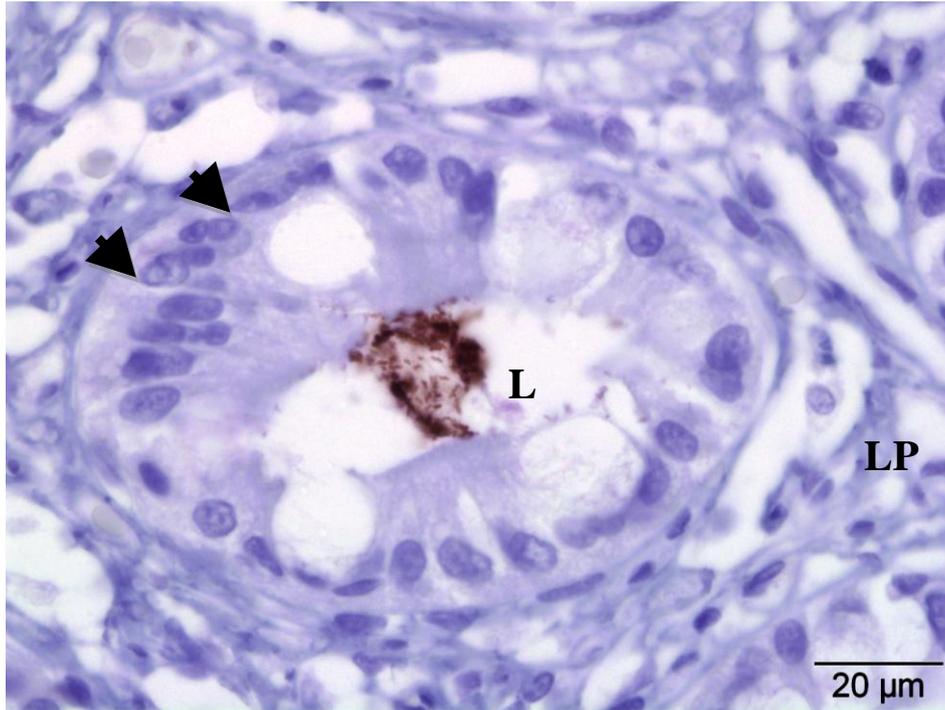


Figure 5-18 Large intestine. Transverse section of large intestinal crypt of dead free-ranging grey seal pup. *Campylobacter* spp. organisms were located on the apical surface of enterocytes and within the intestinal crypt lumen (L). Mild crypt dysplasia characterised by jumbled enterocytes (arrowhead) and mild inflammation of the lamina propria (LP). IHC for *Campylobacter jejuni* counterstained with Mayer's haematoxylin. x600.

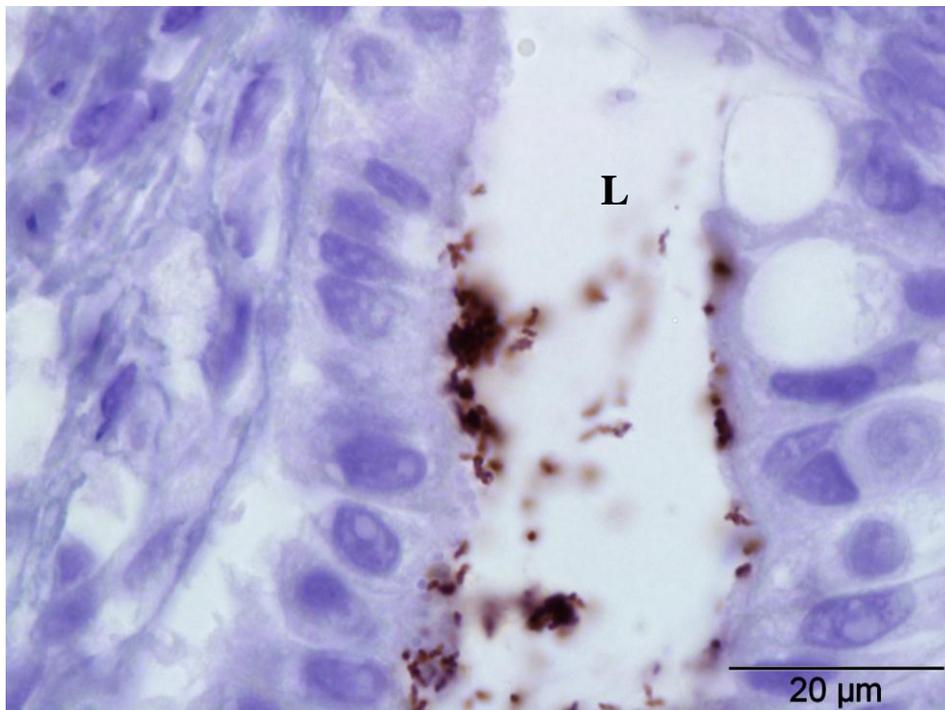


Figure 5-19 Large intestine. Large intestinal crypt of dead free-ranging grey seal pup showing *Campylobacter* spp. organisms located on the apical surface of enterocytes and within the intestinal crypt lumen (L). IHC for *Campylobacter jejuni*. counterstained with Mayer's haematoxylin x1000.

5.4 Discussion

This is the first report of *Campylobacter jejuni*, *lari* or *coli* from any grey seal and the first study to investigate *Campylobacter* spp. of marine mammal origin using whole genome sequencing.

- **Prevalence in free-ranging grey seals**

This study found very high and similar prevalences of *Campylobacter* spp. in both dead and live free-ranging grey seal pups on the Isle of May colony (48%, 95% CI: 34.8,61.5 and 51.1%, 95% CI: 41.0,61.2%, respectively). Although it is difficult to extrapolate between species, the infection prevalence is higher than that previously reported in Northern Elephant seal pups on their natal beaches (13.3%), yet similar to that reported in stranded Northern Elephant seal pups in the same paper (48.5%) (Stoddard et al. 2005). Furthermore, it is substantially higher than the prevalence found in Antarctic fur seals at Deception Island (14.6%) where sympatric Weddell seals (*Leptonychotes weddellii*) were totally free from *Campylobacter* spp. infection (0%) (Garcia-Pena et al. 2010). Given the wide age range of the animals sampled at Deception Island this comparison should be considered with caution.

However, our findings suggest that grey seals are permissive to *Campylobacter* spp. infection as pups due to the high prevalence found in this age group. This is similar to the extensive exposure found in both terrestrial livestock and humans in developed countries (Allos 2001; Grinberg et al. 2005).

The lack of *Campylobacter* spp. in samples from yearlings most likely indicates clearance of these bacteria from the gastrointestinal tract by 1 year of age. However, it is not possible to exclude the possibility that the yearlings sampled in this study had never been exposed to *Campylobacter* spp. or whether they were infected but simply not shedding the bacterium. It noteworthy that *Salmonella* spp. were not isolated from any of these yearling samples either

(see Chapter 4), suggesting a similar clearance process. To investigate the hypothesis that *Campylobacter* spp. are cleared by 1 year of age, further work is required including:

- 1) a mark and recapture approach (longitudinal study), repeatedly sampling a cohort of pups throughout the course of the first year of life and/or
- 2) the prevalence of *Campylobacter* within a population of grey seals could be compared prior to weaning and at 1 year of age the following year, effectively randomly re-sampling individuals from the same population. However, given the poor survival rate of grey seals from birth to 1 year of age (Hall et al. 2001), this process would introduce an inevitable sampling bias towards healthy individuals.

Live grey seal pups on the tidal boulder beach had a significantly lower prevalence of *Campylobacter* spp. than pups on other sites. The reason for this is unclear. Based on results of *Salmonella* spp. culture (Chapter 4) and PhHV1 PCR (Chapter 6), transmission of infectious disease is assumed to be favoured on this site due to the twice daily increased animal density at high tide but this does not seem to apply to this pathogen. Conversely, the alternating exposure to sea-water and periods of desiccation may alter environmental survival and transmission of *Campylobacter*, all the more so given the strict culture requirements of *Campylobacter* discussed previously. It is of note that *Campylobacter* spp. have been recovered from sand samples taken from UK beaches with an overall prevalence of 45% and although the prevalence of *Campylobacter* was higher in wet sand, surprisingly, more than 30% of samples from dry sand also contained these organisms (Bolton et al. 1999). This suggests that *Campylobacter* spp. exhibit a certain tolerance to seawater but little information is available regarding the subsequent ability to form colonies and how this may affect transmission/colonisation of a new host.

- **Prevalence of *Campylobacter* spp. in stranded grey seal pups**

The much lower prevalence of *Campylobacter* spp. in stranded grey seal pups (11.1%, 95% CI: 5.7, 43.5 and 12.5%, 95% CI: 5.0, 28.1, in dead and live seal pups respectively) was unexpected. This differs from the situation described in northern elephant seals where stranded pups were shown to be 6 times more likely to test positive for *Campylobacter* spp. than natal-site seals (Stoddard et al. 2005). However, care must be taken in interpreting this finding as the lower prevalence may be a reflection of the delay in processing swabs rather than a true biological difference. Delay in processing samples and inappropriate swab storage temperatures can significantly decrease the sensitivity of *Campylobacter* isolation (Butt & Wale 1990). The pilot study performed (section 5.2.1) prior to sampling seals suggested that overgrowth by non-specific bacteria may also contribute to this process.

- **Pathogenicity of *Campylobacter* spp. in grey seals**

The similar prevalence of *Campylobacter* spp. in both live and dead, free-ranging pups suggests a non-fatal infection, but does not distinguish between asymptomatic carriage and a chronic-non-fatal condition leading to morbidity.

However, the presence of *Campylobacter* spp. correlated with histopathological lesions such as colitis and crypt abscessation, which are both associated with *Campylobacter* infection in humans and several domestic animal species ultimately leading to diarrhoea and malabsorption of nutrients (Boosinger & Powe 1988; Brown et al. 1999; Macartney et al. 1988). In addition to this, *Campylobacter* spp. were demonstrated immunohistochemically within colonic crypts of infected pups, within the lumen and in close association with the apical surface of enterocytes. The lack of invasion into enterocytes suggests that *Campylobacter* retains a relatively superficial effect which nonetheless could lead to significant faecal fluid loss and dehydration

by acceleration of intestinal transit and malabsorption (Gelberg 2007). However, only a small number of sections were processed for IHC.

It is important to note, however, that correlation does not necessarily imply causality and that the presence of *Campylobacter* spp. may be favoured or facilitated by co-infection with a number of other pathogens or by pre-existing gastro-intestinal pathology. Pathogenicity in terrestrial animals has been established through experimental infections of dogs, ferrets, immunodeficient mice and gnotobiotic piglets (Boosinger & Powe 1988; Hodgson et al. 1998; Macartney et al. 1988; Nemelka et al. 2009). The correlation of *Campylobacter* spp. isolation in rectal swabs with the presence of moderate to severe colitis and immunohistochemical demonstration of *Campylobacter* spp. within crypts is strongly supportive of pathogenicity.

- ***Campylobacter* species**

A wide range of different species and sequence types of *Campylobacter* were isolated from grey seals in this study. The major known zoonotic species *C. jejuni* and *C. coli* predominated, whereas *C. lari*, more frequently associated with seagulls and other migratory birds, was infrequent. The relative proportion of the three *Campylobacter* species found in grey seal pups mirrors both that found in human clinical cases and that found in environmental and food isolates in Scotland (CaMPS/FSA Scotland 2009).

These findings do not support the initial hypothesis that *C. insulaenigrae*, the putative marine mammal specific species of *Campylobacter*, would be present in grey seals. In UK waters, *C. insulaenigrae* has only been reported in harbour seals and a harbour porpoise (Foster et al. 2004). Grey seals may not be permissive to this bacterium or the neonatal grey seals in this study may not have been exposed to it yet.

- **Sequence Types**

The large number of different *C. jejuni* and *C. coli* sequence types (STs) in these grey seal pups support a multifactorial origin rather than circulation of a single, possibly seal adapted, clone of *Campylobacter*. Furthermore, the clustering of isolates, with recently expanded lineages (“clones”) supports the possibility of several independent and recent introductions of *Campylobacter* within the grey seal pup population. Neonatal pups with relatively bacteriologically naïve, newly colonised intestinal flora and a functional yet immature immune system are prime candidates for colonisation, representing effectively a vacant niche for these bacteria. The lack of obvious adaptation or divergence seen between isolates from seals and agricultural sources is also consistent with the hypothesis that seal contamination comes from external sources.

The individual sequence types of *C. jejuni* and *C. coli* are of particular relevance and may help elucidate the origin of these bacteria. The clustering of *C. jejuni* isolated from seals with agricultural *Campylobacter*, indicated very recent divergences between them and would point towards a terrestrial origin of these *Campylobacter* spp. in grey seals.

The most frequent sequence type in the present study was ST-45 (of the ST-45 clonal complex) identified in 7.2% pups (13 of 181). *C. jejuni* isolates belonging to ST-45 are relatively common in a large range of host species (Sheppard et al. 2009a). Based on phylogenetic attribution, 14 ST-45 isolates in the present study formed a “clone” while 3 isolates demonstrated a degree of divergence. In addition the isolate corresponding to ST-1326 was more closely related to ST-45 isolates from seals than other ST-45s from some other species and three other ST-45 isolates from grey seals in this study, which further demonstrates the diversity of *Campylobacter* isolates within sequence types.

Isolates belonging to ST-21 clonal complex were also numerous. ST-21s are also widespread, present in most food animals in Scotland but less commonly in wild birds (Sheppard et al. 2009a). ST-22 was found in 10 seal pups and formed a distinct epidemic clone. This ST is relatively uncommon and has been suggested to be associated with Guillain-Barré syndrome (Revez et al. 2011). It has been most frequently isolated from wild birds and environmental samples (Sheppard et al. 2009a) and is therefore more likely to originate from a wildlife source than the other isolates found in this study. This ST was detected only in pups on the North of the Isle of May breeding colony, which may reflect very localised introduction and spread of this particular pathogen, possibly by sea birds which frequent the island.

All *C. coli* isolates in this study belonged to the ST-828 clonal complex, which is overwhelmingly the most common clonal complex of this *Campylobacter* species in human clinical submissions and which is strongly suggested to represent agriculture-associated disease (488). To the author's knowledge, *C. coli* has not been reported, to date, in marine mammals and is not frequently associated with wild-birds or environmental samples. The presence of this pathogen adds further support to the terrestrial origin of these *Campylobacter* spp. in grey seals.

The two *C. lari* isolates represent two novel sequence types of this species and will be submitted to the pubMLST database.

Source attribution suggests that the *C. jejuni* isolated from seal pups in this study share the same, or very similar, origin as a random sample of 1298 *C. jejuni* isolated from humans. The origin of *Campylobacter* in humans is the subject of substantial on-going research. However, strong evidence implicates chicken meat as the principal source of *C. jejuni* and *C. coli* infection in humans, with the majority of the remaining isolates attributed to ruminant sources (Sheppard et al. 2009b; Strachan & Forbes 2010; Wilson et al. 2008). The overall contribution of wild-

bird, environmental, swine and turkey sources to human clinical cases of Campylobacteriosis is small (Sheppard et al. 2009a; Wilson et al. 2008).

Source attribution and source probability studies of *C. jejuni* in human and grey seal populations demonstrated a stronger contribution of a wild bird origin for *C. jejuni* in seals than in the human population. Similarly the case for chicken being the primary source, whilst highest in both seal and humans, was stronger in the human population. In both populations ruminants maintained a small but significant probability of being the source population.

Finding that seal pups and humans share *Campylobacter* originating from the same sources is a significant and unusual finding. Many wildlife studies have shown *Campylobacter* isolates to be relatively species specific often branching away from known human and terrestrial animal strains forming “wildlife and water” clusters (Hepworth et al. 2011; Stabler et al. 2013). The theory of niche segregation is becoming widely accepted with substantial genetic variation recorded between isolates from different host species (Colles et al. 2008; McCarthy et al. 2007; Sheppard et al. 2010b; Sheppard et al. 2011). This niche segregation does not appear to hold true with regard to the isolates present in grey seal pups in the present study. Indeed the sequence types and clonal complexes of *Campylobacter* in the present study are similar to human rather than environmental/wildlife lineages.

The *Campylobacter* spp. isolates found in this study are likely to originate from the same sources as those found in humans and given that the majority of human cases are thought to be food borne, this raises several questions. Firstly, how is *Campylobacter* initially transmitted to the grey seal population? What role does terrestrial run-off from farming activity and sewage play in the ecology of this pathogen? Finally, could humans themselves be the source of *Campylobacter* for these grey seals?

The lack of geographical clustering of STs or clonal complexes within the colony raises questions as to the route of introduction and spread within the colony. Transmission may occur from several sources: intra-species transmission from infected adult grey seals; interspecies transmission from other species resident on the colony (rabbits, mice or wild birds) or from environmental sources such as water or sediment.

The prevalence of *Campylobacter* in adult grey seals is as yet unknown, although samples taken from the 19 yearlings failed to identify *Campylobacter* in these animals, suggesting that the prevalence is low. The wild birds on the colony were not sampled during this project. Although extrapolating from previous studies in wild birds (Griekspoor et al. 2013; Rutledge et al. 2013), gulls would be expected to carry more host or niche adapted isolates of *Campylobacter* species than were found in grey seal pups in this study. However, given that gulls were frequently seen to scavenge carcasses of grey seal pups, it would have been difficult to interpret the significance of isolating specific *Campylobacter* STs from them. In addition, through this scavenging behaviour, gulls may have facilitated transmission of *Campylobacter* spp. within the grey seal breeding colony, whether acting as a fomite or an intermediate host.

Potential environmental sources include sediment and water. As discussed previously, *Campylobacter* spp. have been widely found in sand from bathing beaches around the UK (Bolton et al. 1999). The major pathogenic species, *C. jejuni* and *C. coli*, were more prevalent in sand from beaches which failed to achieve the minimum EU standards. In contrast, *C. lari* and urease positive thermophilic *Campylobacter* spp., which tend to be associated with seagulls and other migratory birds, were more prevalent in sand from beaches which met with EU standards (Bolton et al. 1999). A higher prevalence of these urease positive *Campylobacter* spp. or *C. lari* would have been expected the present study due to the preponderance of gulls on the Isle of May but other factor must be at play.

Although humans are not a known reservoir for *Campylobacter* spp., the possibility that they represent a secondary reservoir or intermediate reservoir has to be considered given the present findings. The Scottish Environmental protection Agency (SEPA) monitors water quality at bathing beaches annually during summer months, the 2012 results of which are illustrated in Figure 5-20. According to SEPA, sewage remains a significant cause of pollution in Scottish coastal waters despite significant improvement of infrastructure and full secondary treatment of all large continuous discharges (Natural Scotland and Scottish Government 2013). Other land-based run-off includes storm overflow due to heavy rainfall which frequently drains to freshwaters and directly to sea in order to prevent flooding as well as diffuse pollution from agricultural and rural sources (Natural Scotland and Scottish Government 2013).

Efforts are underway to further develop sustainable urban drainage systems (SUDS), which include permeable surfaces and artificial ponds or wetlands aiming to act as natural filters to pathogens and toxins (Natural Scotland and Scottish Government 2013).

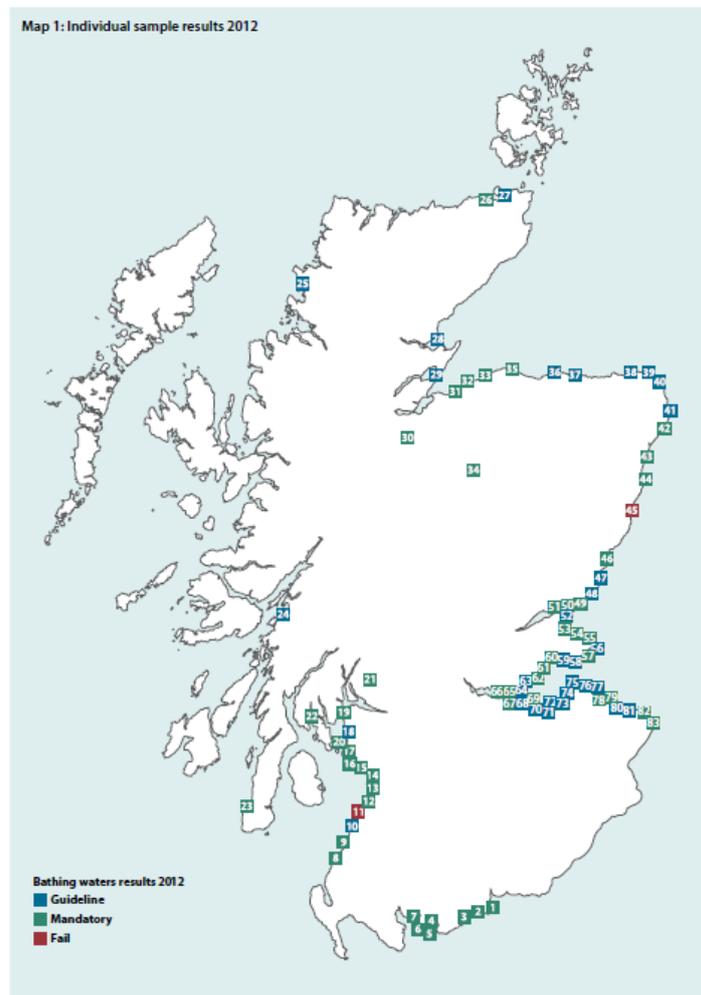


Figure 5-20 Bathing water results 2012. Scottish bathing water monitored by SEPA identifying waters achieving guideline quality for EU compliance; mandatory guideline for EU compliance and waters failing the guideline for EU compliance. Origin: Scottish Environment protection Agency (SEPA) (Natural Scotland and Scottish Government 2013)

The use of genomic analysis in the present study has the advantage of providing rapid identification and high resolution comparisons between *Campylobacter* isolates. This technology is useful, in addition to more conventional molecular approaches such as MLST, to improve resolution among *Campylobacter* clonal complexes and between very closely related isolates (Cody et al. 2013). As the number of *Campylobacter* isolates increases within known sequence types, distinction between individual isolates will become more relevant, relying more heavily on comparison of other genes and processes such as whole genome MLST (Cody et al.

2013; Sheppard et al. 2012). The 90 genomes of *Campylobacter* spp. acquired throughout the present study will contribute to the extensive pubMLST database in the future.

- **Phenotypes**

Campylobacter phenotypes have been shown to be diverse even within a same sequence type. In combination with the unreliable nature of phenotypical assays there is increased reliance on molecular methods in identification of *Campylobacter* spp. (Steinhauserova et al. 2001; Stoddard et al. 2007).

The discrepancy between the results of the two successive hippurate hydrolysis assays emphasises the notorious variability that can be obtained using this test (Rautelin et al. 1999; Totten et al. 1987). Increasing the overall concentration of inoculum and reducing processing delay to a minimum results in much more reliable results and is undoubtedly a reflection of the exquisitely sensitive nature of this pathogen to temperature and oxygen among other parameters.

5.5 Conclusion

This study demonstrated that free-ranging grey seal pups on the Isle of May and from around the Scottish coastline are exposed to a wide range of *Campylobacter* spp. the origin of which is likely to be the same as that of human clinical infections. This outcome is contrary to the hypothesis that seals are more likely to acquire such isolates from environmental sources or wild birds and supports the possibility of land-sea transfer of this enteric pathogen from terrestrial animal sources to grey seals.

Beyond the direct effect on grey seals, degradation of water quality of coastal habitats may have downstream implications on human health and well-being.

Chapter 6 The prevalence of known viral and protozoal pathogens in neonatal and juvenile grey seals (*Halichoerus grypus*) around the Scottish coastline: a surveillance-based approach

6.1 Introduction

Reports of infectious disease in marine mammals have significantly increased in recent years (Gulland & Hall 2007). To understand whether this signifies that marine mammal health is declining, due to factors such as increased anthropogenic pressures or climate change, or whether this is simply a reflection of increased sampling effort and newly available technologies, baseline studies and directed marine mammal health monitoring programs are needed (Bossart 2011; Gulland & Hall 2007; Hall et al. 2010; Reddy et al. 2001).

A long list of pathogens has been found in grey seal populations, generally associated with disease conditions (Miller et al. 2001a). Some are relatively well understood, such as the paramyxovirus phocine distemper virus (PDV), through its similarity with canine distemper and dramatic impact on harbour seal (*Phoca vitulina*) populations which elicited much research effort (Barrett et al. 1992; Hall et al. 1992; Osterhaus 1989). Grey seals were suspected to be asymptotically infected with PDV, leading to complex theories of two or three host systems with regard its epidemiology (Hall et al. 2006; Hammond et al. 2005b; Pomeroy et al. 2005). Other agents, such as phocid herpesvirus 2 (PhHV2), have been found in apparently healthy animals or in animals with underlying disease conditions, leaving large gaps in our understanding of the significance of this infectious agent (Martina et al. 2003).

The grey seal population in the North sea is still growing exponentially, but in other regions, such as the Outer Hebrides and Orkney, this exponential growth rate has stopped in recent years (Sea Mammal Research Unit 2009). Population dynamics models indicate that this is due to density dependence which is acting through reduced pup survival rather than a decrease in female fecundity (Hall et al. 2001; Lonergan et al. 2011a). This implies that females are giving birth but pups are not surviving. However, the ultimate cause of death in these pups is very poorly understood. It is predicted that a similar pattern of population is likely to occur in the North Sea colonies, including the Isle of May (Sea Mammal Research Unit 2009). To fully understand the population dynamics in grey seals and the processes which influence this, the drivers of changes in population trends need to be understood. Therefore knowledge of the pathogens present and causes of mortality and morbidity in grey seal pups will greatly aid understanding of the contribution that these disease agents may have in shaping future population trends and trajectories.

Additionally, determining what constitutes a “normal” baseline burden of disease in grey seal populations will help understand the routine threats to neonatal grey seals and provide some idea of which diseases are likely to appear or be exacerbated by stress or malnutrition at rehabilitation centres.

Furthermore, understanding the prevalence of different pathogens, in particular given the potential role of grey seals as asymptomatic carriers for pathogens such as PDV (Hall et al. 2006), may help understand threats to other, more elusive, sympatric species such as the common seal, the populations of which are in dramatic decline in several areas of Scotland (Lonergan et al. 2007).

Disease exposure is often measured using techniques such as serology which detects specific antibody to a particular pathogen, indicates that an animal has been exposed to each specific pathogen and that this animal has subsequently mounted an immune response. However, except in the acute phase of infection, serology gives little indication of the likely time point of exposure, or if pathology developed and the animal had been compromised in any way. Furthermore, in neonatal animals serology is complicated by the presence of maternal antibodies which can be transferred to the offspring transplacentally or via the colostrum. Thus, serological evidence of exposure to a pathogen may reflect prior exposure of the dam rather than the pup. Conversely, in acute infections, a negative result may occur if seroconversion has not had time to occur. Establishing the presence of specific pathogens at the time of sampling calls for techniques which detect the pathogen itself such as polymerase chain reaction (PCR), viral or bacterial culture methods, immunohistochemistry (IHC), histology or electron microscopy to visualise or detect the pathogen in tissues or body fluids. The detection of each pathogen does not imply pathogenicity, merely presence and needs to be considered in parallel with clinical status in live animals or a rigorous gross and histopathological post-mortem examination.

The **aims of this study** were two-fold:

- 1 - To assess the prevalence and risks of viral and protozoal pathogens to grey seals, with particular emphasis on pup survival and morbidity.
- 2 – To use grey seals as sentinels of coastal marine pollution arising from pathogens of terrestrial animal origin.

A **broad surveillance approach** was used to assess viral and protozoal exposure in stranded and free-ranging grey seal pups and yearlings. **Pathogens of interest** included seal pox virus, phocid herpesvirus 1 (PhHV1), phocine distemper virus (PDV); phocid herpes virus 2 (PhHV2), *Neospora caninum* and *Toxoplasma gondii*. The prevalence and significance of the bacterial

pathogens *Brucella* spp., *Listeria monocytogenes*, *Campylobacter* spp. and *Salmonella* spp. are covered separately in Chapters 3, 4 and 5. The presence of these pathogens was investigated using a variety of different methodologies including polymerase chain reaction (PCR), bacterial culture, histopathology and immunohistochemistry.

Diseased, malnourished and stressed animals are considered more likely to be immunosuppressed and by this assumption are more likely to harbour higher numbers of infectious agents. In light of this, seal pups dying on the Isle of May are likely to have a higher prevalence of pathogens than overtly healthy pups from the same breeding locations within the Isle of May colony. In addition the present study investigates whether there was a difference in spatio-temporal distribution of pathogens, in particular PhHV 1.

In general, animals of different ages harbour different commensal organisms and are affected by different pathogens. It is therefore likely that live, overtly healthy yearling grey seals will carry different nasal and rectal pathogen burdens than overtly healthy grey seal pups on the Isle of May colony. To investigate this, the prevalence of nasal and rectal pathogens was compared between stage I pups, stage V pups and yearlings captured on the Isle of May.

“Stranded” grey seal pups are assumed to be stressed and are frequently malnourished, both factors known to impair immune function (Snyder 2007). This would result in pups being more susceptible to infection and prolong existing infections. Consequently, the prevalence of known endemic grey seal pathogens, other than pathogens of anthropogenic origin, is likely to be higher in stranded grey seal pups than in grey seal pups from their natal colonies. In order to investigate this, the prevalence of a known seal pathogens, PhHV1, in seal pups on admission to the Scottish SPCA National Wildlife Centre was compared to that of live seal pups sampled on the Isle of May colony.

6.2 Materials and methods

6.2.1 Samples

Grey seal pups and yearlings were sampled as part of an extensive screening effort during the pupping season of autumn 2011. Free-ranging, dead (n=50) and live (n=90) grey seal pups and live yearlings (n=19) were sampled on the Isle of May, Firth of Forth, UK. Stranded live grey seal pups (n=31) and stranded pups that died or were euthanised on humane grounds (n=9) were sampled from the Scottish SPCA National Wildlife Centre, Fife.

Sampling of each group was performed as detailed in Chapter 2.

All sampling of live animals on the Isle of May was carried out under UK Home Office Project (No. 60/4009) and Personal Licences as issued to the Sea Mammal Research Unit under the Animals (Scientific Procedures) Act, 1986. All sampling of live animals submitted to the rehabilitation centre was for diagnostic purposes to determine the cause of stranding and future treatment regime.

6.2.2 Sample analysis / processing

- **“Viral” tissue pools and individual tissue samples for virology**

Pooled tissue samples (11 organs) (see section 2.3) and individual tissue samples (12 organs) were homogenised with 5ml viral transport medium (Hanks balanced salt solution containing 0.02% phenol red, 1% w/v bovine serum albumin, 0.45 w/v sodium hydrogen carbonate, 600 µg/ml benzyl penicillin, 0.3 mg/ml streptomycin sulphate, 50 µg/ml polymyxin B and 50 µg/ml nystatin), using a Dispomix (Miltenyi Biotec Ltd., Bisley, UK) homogeniser at a predefined profile (two cycles of 3 seconds (s) at 2000rpm (rotations per minute) followed by two cycles of

3s at 4000rpm, two 3s cycles at 2000rpm and two 3s cycles at 4000rpm, each cycle separated by a 3s pause and the sense of rotation changed at each successive cycle); centrifuged at 2000g for 10 minutes (min) at 4°C and the resultant supernatant transferred to a sterile microcentrifuge tube and stored at -80°C until required for further analysis.

- **Apicomplexan tissue pools and individual tissue samples**

Individual samples of tongue, lung, brainstem and heart, along with a pooled sample of these four tissues, were thawed at room temperature, finely chopped using sterile scalpel blades and transferred to a Precellys® tissue homogeniser tube (Cepheid, Stretton Derbyshire, UK) containing 1 ml nuclei lysis solution (Promega, Madison WI, USA) and homogenised for 2x 50s at 6500 rpm (Precelys 24 tissue homogeniser) (Cepheid, Stretton Derbyshire, UK).

- **Nasal swabs**

Nasal swabs in universal transport medium (Cat no. 346C, Sterilin, Newport, UK) were placed into a sonicator bath for 30s and then centrifuged at 2000g for 10min at 4°C. The resultant supernatant was transferred to a sterile microcentrifuge tube and stored at -80°C until required for further analysis.

- **Buffy coats**

Buffy coats were prepared by centrifugation of heparin treated whole blood at 2000g for 10min; contaminating erythrocytes were lysed twice with 0.017 M Tris, 0.014 M ammonium chloride buffer, pH7.2 and buffy coats harvested using a Pasteur pipette. Particular care was taken to prevent contamination of the samples, intended for downstream PCR. The resulting cell pellet was washed and resuspended in sterile PBS prior to storage at -80°C.

6.2.3 Nucleic acid extraction

Nucleic acids of all nasal swabs, buffy coat preparations, homogenised “viral” tissue pools and corresponding individual tissue samples for virology were extracted using the NorDiagViral NA Arrow automated extraction robot (Alere, Stockport, UK) as per manufacturer’s instructions.

DNA of the tissue pools and corresponding individual tissue samples for investigation of apicomplexan protozoa was extracted as described by Bartley *et al.* (Bartley *et al.* 2013). In brief, lysed tissue homogenate was processed to DNA using the Wizard genomic DNA purification protocol (Promega UK, Southampton, UK) and scaled up to allow for 0.4 g of starting material. The DNA was resuspended in 200 µl of DNase/RNase free water and stored at -20 °C prior to PCR analysis.

Extraction controls (water or PBS) were processed with every batch of tissue samples processed for DNA extraction. These extraction controls were used to assess contamination and acted as additional negative controls for the nested PCRs. All nucleic acid extractions were stored at -20°C prior to testing.

6.2.4 PCR assays

- **Real time PCR (qPCR)**
 - **Beta-actin qPCR to control for RNA amplificability (endogenous internal control)¹⁰**

A β-actin primer and probe set was used in a separate assay to assess sample integrity and RT-qPCR inhibition. The primer and probe sequences, used are shown in Table 6-1.

¹⁰ Beta-actin qPCRs were carried out by JB and JC

All eluted nucleic acid from the pooled and individual tissue samples and mononuclear leucocytes was assessed for RNA and cDNA amplificability using a reverse transcriptase real-time PCR (RT-qPCR) assay amplifying the stable housekeeping gene beta-actin (Thonur et al. 2012). Bovine embryonic kidney primary cells were used as a positive control.

In addition, nucleic acid samples from nasal swabs that were negative on end-point PCR for glycoprotein B of PhHV1 were assessed for amplificability using the same qPCR protocol to validate the lack of PhHV1 detection.

All samples that resulted in a negative test for β -actin were re-extracted (as above) and re-assessed for nucleic acid amplificability. Any samples that were repeatedly negative for β -actin were excluded from the analyses which depended on these samples.

- **Morbillivirus¹¹**

All eluted nucleic acid from the pooled tissue samples was assessed for the presence of PDV using a real-time PCR assay amplifying a 115 bp fragment of the PDV H gene (Hammond et al. 2005b). The primer and probe sequences, used are shown in Table 6-1.

Positive control material (lung from a harbour seal known to be infected with PDV during the 2002 outbreak) was kindly provided by the Sea Mammal Research Unit (SMRU) at the University of St Andrews.

- **Parapoxvirus**

All eluted nucleic acids from the “viral” pooled and individual tissue samples were assessed for the presence of Parapoxvirus DNA using a real-time PCR assay amplifying a 95bp fragment of

¹¹ Morbillivirus qPCRs were carried out by JB and Jacob Chapman as part of a Wellcome Trust summer studentship at MRI

the highly conserved major envelope protein gene, B2L (Nitsche et al. 2006). The primer and probe sequences, used are shown in Table 6-1. Positive control material (DNA extracted from cell cultures known to be infected with Parapoxvirus isolated from a skin scab of a grey seal) was kindly provided by Dr C. McInnes, MRI (VSU virus strain BJ 820).

- **qPCR Parameters**

Optimisation of the parapoxvirus qPCR included testing several master mix reagent sets, titration of primer and probe concentrations and alteration of PCR cycling parameters¹². For each primer/probe combination working concentrations were optimised to ensure optimum conditions were used. A checkerboard assay of primers at 300 nM, 600 nM, and 900 nM was used to optimize the real-time protocol. Probes were subsequently titrated at several levels from 50 to 200 nM final concentration with the optimized levels of primers.

Efficiency of the optimised qPCR assays were evaluated by performing a 10 fold dilution series (Figure 6-1) of a positive control DNA/RNA as appropriate.

¹² optimisation of the PDV RT-qPCR had previously been carried out by Amy Finlayson as part of a studnet summer project (unpublished); Johanna Baily carried out the optimisation of the parapoxvirus qPCR

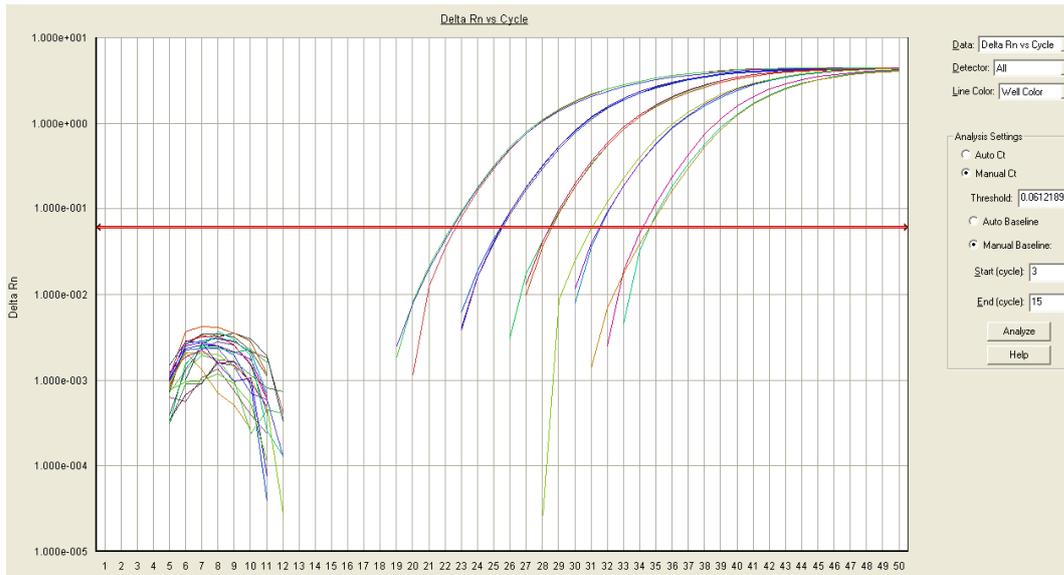


Figure 6-1 Amplification plot of ten-fold dilution series of parapoxvirus positive control (seal pox virus) using optimised primer/probe concentrations

Superscript III RT/Platinum Taq mix (Invitrogen, Paisley UK) and Quanta Perfecta qPCR Supermix Low ROX (Quanta Biosciences, Inc., Gaithersburg, USA), were chosen as the optimum reagents for RT-qPCR (PDV and β -actin) and qPCR (parapoxvirus) respectively. Reactions were performed in triplicate using a total volume of 25 μ l containing a final concentration of 300 nM each primer, 50 nM each probe and 2 μ l of template RNA. The β -actin RT-PCR was performed in a similar manner except that final primer and probe concentrations were 500 nM and 200 nM respectively. All reactions were performed on an ABI Prism 7500 (Applied Biosystems, Warrington, UK) with the following cycling parameters for RT-qPCR: 50°C for 30 min (RT) and 95°C for 2 min (hot start), 45 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension). For qPCR, cycling parameters were as follows: 95°C for 2 min (hot start), 50 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension).

Table 6-1 Probes and primers used for real-time PCR assays. All the primers and probes were obtained from Applied Biosystems (Warrington, UK). Abbreviations: MGB-NFQ minor groove binding-non fluorescent quencher; PPV: Parapoxvirus

Target	Working conc.	Primers/probes	Sequences (5' - 3')	Reporter dye
Mammalian Beta actin	200 500 500	BacMGB_mammal Bac1F_uni Bac2R_uni	NED-TGA AGA TCA AGA TCA TCG-MGB-NFQ GAC AGG ATG CAG AAR GAG ATC AC TCC ACA TCT GCT GGA AGG TG	NED
PDV	50 300 300	PDV-probe-FAM PDVrt F PDVrt R	FAM-CAT GTC CCT CAT ATC AAA ACC TTC GGA GG-TAMRA ACC TCG ATG GGC AAT GTC TT GTC TTA CCG TAG ATC CCT TCT GAG AT	FAM
PPV	50 300 300	PPVTMGB PPVup PPVdo	FAM-TGC GGT AGA AGC C-MGB-NFQ TCG ATG CGG TGC AGC AC GCG GCG TAT TCT CGG AC	FAM

The results were analysed using Applied Biosystems Sequence Detection Software Version 1.4 (Applied Biosystems) as per the manufacturer's instructions.

- **End-point (conventional) PCR assays**

- **Phocid herpesvirus 1**

Nucleic acid from the nasal swabs and pooled tissue samples was assessed for the presence of PhHV1 using an end-point PCR assay amplifying a 450bp fragment of the PhHV1 glycoprotein B (gB) gene. PCR primers and amplification conditions were those used previously (Goldstein et al. 2004) and detailed in Table 6-2. DNA extraction controls and positive (PhHV-1 DNA extracted from infected cell cultures¹³) and negative (no template DNA) controls were included

¹³ Sample of grey seal liver was positive for PhHV1 using pan-herpesvirus nested degenerate polymerase chain reaction (PCR) method, targeting the DNA polymerase gene and incorporating inosine substituted primers (Ehlers et al. 1999). Direct sequencing (Eurofins MWG, Ebersberg, Germany) was performed and sequence similarities were determined using BLAST (Altschul et al. 1997) (<http://blast.ncbi.nlm.nih.gov>).

for all reactions. Reactions were performed as per the manufacturer's protocol in a total volume of 50 µl, using Platinum Taq (Invitrogen) and containing a final concentration of 200 µM of each primer and 2 µl of template DNA.

All reactions were performed in a Techne Workbench thermal cycler (Techne, Stone, UK) with the following cycling parameters: 95°C for 3 min (denaturation/Taq activation), followed by 35 cycles of denaturation for one minute at 94°C, annealing for one minute at 60°C, and extension for one minute at 72°C, with a final extension at 72°C for 10 minutes. Reaction products were electrophoresed on a 1.5% agarose gel, stained with Sybersafe (Invitrogen) and visualised by UV transillumination using a gel doc system (Alpha Imager).

Table 6-2 Primers used for end-point PCR assays

Target	Primers	Sequences (5' - 3')	Expected product size
gB of PhHV1	PhHV-1 gB-F PhHV-1 gB-R	ACGTGATGGAGCTCATACAGAAC GCTAGCTCGTTGCTAATCATTGG	450bp
UL52 of PhHV2	PhHV-2 UL52 F PhHV-2 UL52 R	TG TTCAGATGCCAGTTCC TTCATCCAAGTCCC ACTC	430bp
Apicomplexan parasite ITS1 region – 1 st round (<i>T. gondii</i> and <i>N. caninum</i>)	NN1 NN2	TCAACCTTTGAATCCAAA CGAGCCAAGACATCCATT	397bp
<i>Toxoplasma gondii</i> 2 nd round	Tg-NP1 Tg-NP2	GTGATAGTATCGAAAGGTAT ACTCTCTCTCAAATGTTCT	227bp
<i>Neospora caninum</i> 2 nd round	Nc-NP1 Nc-NP2	TACTACTCCCTGTGAGTTG TCTCTTCCCTCAAACGCT	279bp

- **Phocid Herpesvirus 2**

Nucleic acids from the pooled tissue samples and buffy coats were assessed for the presence of PhHV2 using an end-point PCR assay amplifying a 430bp fragment of the UL52 gene of the PhHV2 strain 7848. PCR primers were those used previously by Martina *et al.* (2003) and detailed in Table 6-2. DNA extraction controls and positive (DNA from PhHV2 infected cells kindly provided by Dr B. Martina, Erasmus MC, Rotterdam, NL) and negative (no template DNA) controls were included for all reactions. Reactions were performed in a total volume of 50 μ l, as per the manufacturer's protocol, using Platinum Taq (Invitrogen) and containing final concentrations of 200 μ M of each primer and 2 μ l of template DNA.

All reactions were performed in a Techne Workbench thermal cycler (Techne, Stone, UK) with the following cycling parameters: 95°C for 3 min (denaturation/Taq activation), followed by 40 cycles of denaturation for 30s at 94°C, annealing for 60s at 60°C, and extension for 60s at 72°C, with a final extension at 72°C for 10 min. Reaction products were electrophoresed on a 1.5% agarose gel, stained with Sybrsafe (Invitrogen) and visualised by UV transillumination using a gel doc system (Alpha Imager).

To confirm PCR specificity for selected suspected positive samples and positive control sample, PCR products were cleaned using the Charge switch PCR clean up kit (Invitrogen), as per manufacturer's instructions and sequenced once in both directions by single read method by Eurofins (MWG Operon, Ebersberg, Germany) using the PCR primers.

Forward and reverse sequence pairs were aligned with CLUSTALW (BioEdit software) using paired reads. For comparison with previously published strains of PhHV2 (Martina *et al.* 2003), consensus sequence reads were aligned using Seaview alignment software and a maximum likelihood analysis was performed on a 384bp portion of the amplicons, generating phylogenetic trees.

○ ***Toxoplasma gondii* and *Neospora caninum* PCRs¹⁴**

Nucleic acids from the pooled lung, tongue, heart and brain tissues and individual tissue samples was assessed for the presence of *T. gondii* and *N. caninum* DNA using a nested PCR assay amplifying 227bp and 279bp fragments of the multicopy 18S-5.8S rRNA internal transcribed spacer 1 (ITS1) region of *T. gondii* and *N. caninum*, respectively.

For *T. gondii*, the nested ITS1 primers NN1 – NN2 and Tg-NP1 – Tg-NP2 were previously described by Hurtado *et al.* (2001) and the PCR was carried out according to the protocol described by Burrells *et al.* (2013). For *N. caninum*, the nested ITS1 primers NN1 – NN2 and Nc-NP1 – Nc-NP2 were previously described by Buxton *et al.* (1998) and the PCR was carried out according to the protocol described by Bartley *et al.* (2013).

The reaction mixture and amplification conditions were as follows. Each reaction (20 µL) consisted of 2 µL of 10× custom PCR master mix, giving a working concentration of 45mM Tris-HCl pH 8.8, 11 mM (NH₄)₂SO₄, 4.5mM MgCl₂, 0.113 mg/mL BSA, 4.4 µM EDTA and 1.0mM each of dATP, dCTP, dGTP and dTTP (ABgene, Surrey, UK), 1 µL each forward and reverse primer (5pmol) (primary amplification- NN1, NN2; secondary amplification- NP1, NP2), 0.15 µL (5U/µL) Taq polymerase (Bioline Ltd. London, UK), 13.85 µL DNase / RNase free water and 2 µL sample DNA, with each sample being analysed in triplicate. The reaction conditions for both primary and secondary amplifications were as follows 95 °C for 5 min followed by 35 cycles at 95 °C for 1 min, 55°C for 1 min and 72 °C for 1 min and a final extension period of 72 °C for 5 min. The primary amplicon was diluted with 100 µL DNase / RNase free water and 2 µL of the diluted product was used as template DNA for the second round amplification. The conditions for each second round were identical to the first using the relevant primers for the assay presented in Table 6-2. Following this, 10 µL of second round

¹⁴ *T. gondii* and *N. caninum* extractions and PCRs were carried out by Paul Bartley, MRI

product was subjected to 2% agarose gel electrophoresis, stained with GelRed™ (Biotium Inc., Hayward, USA) and visualised by UV light. A sample was considered positive if one of the triplicates gave a visible band of 227bp (for *T. gondii*) or 279bp (for *N. caninum*).

Each PCR run contained a positive control (DNA extracted from tissue culture derived *T. gondii* or *N. caninum* tachyzoites depending on the assay) and multiple negative control (water and extraction control) samples per 96-well plate. Results were only accepted if the positive and multiple negative controls gave appropriate results.

6.2.5 Histopathology and IHC

- **Histopathology**

As described in Chapters 2 and 3, samples of 36 different tissues were routinely taken from all 59 pups submitted for post-mortem examination, fixed in 10% neutral buffered formalin and processed routinely for histopathological examination. All histopathology was interpreted by a single pathologist (JB).

- **Immunohistochemistry¹⁵**

Tissue sections (5 µm) were placed on Superfrost Plus slides (Thermo Fisher Scientific). All sections were dewaxed in xylene and rehydrated through 100%, 75%, and 50% ethanol. Antigen retrieval was performed as summarised in Table 6-3. Endogenous peroxidase activity was blocked by immersion in 3% H₂O₂ in methanol (vol/vol) for 20 min. Tissues were rinsed with running tap water for 5 min and then incubated with 150 µl of 25% normal goat serum (NGS; Vector) in PBS-0.05% Tween 20 (PBST) for an hour at room temperature to block non-specific antibody binding.

¹⁵ Immunohistochemistry for Bovine herpes virus 1 and *Toxoplasma gondii* was performed by Clare Underwood, MRI; IHC for Feline Herpesvirus 1 was performed by Marion Stevenson, University of Glasgow.

Primary antibodies to various pathogens were: Bovine herpesvirus 1 (clone F2; mouse monoclonal IgG2b (Veterinary Medical Research and Development, Pullman, WA, USA), dilution 1:5000), feline herpesvirus 1 (Clone FHV5; mouse monoclonal IgG2b (Acris antibodies, Herford, Germany), dilution 1:100) and *Toxoplasma gondii* (*T. gondii* tachyzoites and bradyzoites (Buxton et al. 1998), rabbit polyclonal, dilution 1:4000), as primary antibodies. The dilutions used, sources of the primary antibodies and antigen retrieval conditions used in this study are described in Table 6-3. Visualisation of the primary antibody was by the appropriate Envision™ Kit (Vector Laboratories etc.) as per manufacturer's protocols.

Table 6-3 Antibodies used in this study

Pathogen targeted	Antibody (Ab) name, Source, type	Antigen retrieval	Primary Ab dilution	Primary Ab incubation	Secondary Ab	Positive control material
α-Herpes virus (PhHV1)	Bovine Herpesvirus Type 1 (BHV-1/IBR), clone F2, Mouse monoclonal gC-gIII IgG2b, (VMRD)	None	1:5000	Overnight 4°C	Envision anti-mouse HRP polymer	Bovine liver infected with BoHV1
α-Herpes virus (PhHV1)	Feline Herpes Virus Type 1 , Clone FHV5, mouse monoclonal IgG2a (ACRIS Antibodies)	None	1:100	30 min RT	Envision anti-mouse HRP polymer	Feline lung infected with FHV1
<i>Toxoplasma gondii</i>	Rabbit polyclonal anti- <i>Toxoplasma gondii</i> (Buxton et al. 1981)	None	1:4000	Overnight 4°C	Envision goat anti rabbit HRP	Mouse liver experimentally infected with <i>T. gondii</i>

6.2.6 Statistical analyses

Prevalence data were analysed using the R statistical software package (R Core Team 2013). To investigate significant differences in prevalence data between groups, Fisher's exact test and Chi-squared tests were performed. For univariate analysis, generalized linear models (GLMs) with a binomial distribution family and a logit link function were used to evaluate the effects of different morphologic measurements (standard length, mass, axillary girth), age (stage of pup development as a proxy), sex, sampling time and location on the presence or absence of each pathogen.

Multivariate logistic regression was performed using R with a forward stepping algorithm and a p value of ≤ 0.05 for inclusion in the model based on the likelihood ratio test. Dead stranded grey seal pups were excluded from the multivariate analysis of PhHV1 nasal swab status to avoid repeat sampling of the individual animals swabbed on entry to the rehabilitation centre.

As length, mass, girth and pup stage were highly correlated with each other (see Chapter 2) and so as not to violate the assumption in generalised linear models concerning the independence of independent variables, mass was chosen as the most reliable, independently verified and reproducible of the morphometric measurements in the present study and retained for further analyses (see Chapter 2 for details).

Akaike's Information Criterion (AIC) was used to compare models and choose the most parsimonious for each variable. Residual analysis was used to assess goodness of fit.

6.3 Results

6.3.1 Beta-actin PCR

Nucleic acid sampled from all but two tissue pools (CD004 and CD037) and one nasal swab (CD031) were positive for β -actin. The three samples that were β -actin negative were all excluded from subsequent analyses on the basis that amplificability of DNA/RNA could not be verified.

6.3.2 Phocid Herpesvirus 1

- **Nasal swabs**

The prevalence of PhHV1 in nasal swabs of all pups was 59.6% (105/176). The group with the highest prevalence was the stranded dead pup group with a prevalence of 88.9% (Table 6-4). Yearlings had a significantly lower prevalence of PhHV1 than pups (27.8% vs 59.0% respectively; $p=0.008$; Fisher's exact test).

A small cohort of 7 stranded grey seal pups were tested for PhHV1 both on arrival at the rehabilitation centre and at the time of death. Of these 5 were positive for PhHV1 on arrival and 2 were negative, yet all 7 pups were positive for PhHV1 at the time of death on nasal swabs. This difference was not statistically significant ($p=0.461$; Fisher's exact test) but the small sample size is likely a limiting factor.

Odds ratios for categorical risk factors were determined for PhHV1 (Table 6-5). Free-ranging pups sampled late in the pupping season had significantly higher odds of shedding PhHV1 in nasal swabs, as determined by presence of PhHV1 nucleic acids, than pups sampled in the early part of the pupping season. On the Isle of May, this was true for both the live free-ranging pups and the free-ranging live and dead pups combined ($p=0.011$ and $p<0.001$, respectively; GLM).

Also, free-ranging pups sampled at stage I were significantly less likely to be shedding PhHV1 in nasal secretions compared to pups in any of stages III, IV and V ($p=0.004$; $p=0.002$; $p=0.024$, respectively).

Table 6-4 - Prevalence of each pathogen. Number of positive animals (%: percentage of each group)

Pathogen	Stranded Live	Stranded Dead	Colony Live	Colony Dead	Colony Yearlings
PhHV1 Nasal swab	18 (62.1%)	8 (88.9%)	52 (57.8%)	27 (56.2%)	5 (27.8%)
PhHV1 Pooled tissue samples		6 (66.7%)		26 (54.2%)	
PhHV2 Pooled tissue samples		0 (0%)		0 (0%)	
PhHV2 Buffy coat					3 (15.8%)*
Morbillivirus pooled samples		0 (0%)		0 (0%)	
Poxvirus pooled samples		1 (11.1%) **		0 (0%)	
<i>Toxoplasma gondii</i> pool		0 (0%)		3 (6%)	
<i>Neospora caninum</i> pool		0 (0%)		0 (0%)	

* minimum estimate based on sequencing results

** R006 – very weakly positive (probably contamination)

All age indices (Pup developmental stage, mass, girth and time of sampling) were positively correlated with PhHV1 nasal shedding, confirming the initial hypothesis was that PhHV1 status was related to age or development stage. As discussed in Chapter 2, these were correlated variables that gave the same development ‘index’. All these factors were included in the initial generalised linear model, yet stepwise analysis revealed mass as the best fitting model using these variables.

Table 6-5 Categorical risk factors, using univariate analysis, for detecting Phocid herpesvirus 1 from nasal swabs of grey seals; (n=: group size; OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: p<0.05; **: p<0.01; ***: p<0.001)

	Risk factor	Group	(n=)	number (%) positive	OR	95% CI	P-value	Sign.	
All seals	Group	Free-ranging - live	90	52 (57.8%)	1.00				
		Free-ranging - Dead	50	27 (56.2%)	0.94	0.46, 1.91	0.863	NS	
		Stranded - Live	31	18 (62.1%)	1.20	0.51, 2.82	0.683	NS	
		Stranded – died/euthanised	9	8 (88.9%)	5.85	0.7,48.73	0.103	NS	
		Live free-ranging yearlings	19	5 (27.8%)	0.28	0.06 0.86	0.025	*	
Free-ranging live and dead pups	Live/dead	Dead	50	27 (56.2%)	1.00				
		Live	90	52 (57.8%)	1.06	0.52, 2.16	0.863	NS	
	Sex	Female	65	34 (52.3%)	1				
		Male	72	45 (62.5%)	1.52	0.77, 3	0.228	NS	
	Mass range (kg)	<12	13	5 (38.5%)	1				
		12-25	73	31 (42.5%)	1.18	0.35, 3.96	0.787	NS	
		25-31	13	11 (84.6%)	8.8	1.35, 57.43	0.02	*	
		>31	39	11 (28.3%)	7.31	1.83, 29.2	0.004	**	
	Free-ranging Live pups only	Time point	Early	30	11 (36.7%)	1.00			
			Mid	30	20 (66.7%)	3.45	1.19, 9.99	0.022	*
Late			30	21 (70%)	4.00	1.37, 11.84	0.011	*	
Sampling site		Tidal boulder beach	30	22 (73.3%)	1.00				
		Rocky pools	30	18 (60%)	0.55	0.18, 1.62	0.276	NS	
		Muddy grassy slope	30	12 (40%)	0.24	0.08, 0.72	0.011	*	
Coat		Stage II	41	16 (39%)	1.00				
		Stage III	13	9 (69.2%)	3.52	0.93, 13.35	0.065	NS	
		Stage IV - Moulting	27	21 (77.8%)	5.47	1.81, 16.48	0.003	**	
		Stage V- Adult coat	9	6 (66.7%)	3.12	0.68, 14.31	0.142	NS	

For live, free-ranging pups, the best fit mixed effects logistic regression model included sampling site and sampling time as fixed effects. The interaction with stage and sampling time could be considered for inclusion in the final model but these factors lose statistical significance when controlling for mass in the final model and were excluded from the final model on that basis.

In the final logistic regression model (Table 6-6), an increased risk for PhHV1 nasal shedding was associated with the location, as well as with the mass of the pup sampled. Seals born on the rocky pools (OR=0.55, 95% CI 0.18, 1.62, $p=0.015$) and on the grassy, muddy slope (OR=0.24, 95% CI 0.08, 0.72, $p=0.002$) were less likely to be positive for PhHV1 in nasal swabs than pups born on the tidal boulder beach. The odds of shedding PhHV1 were increased by a factor 1.15 for every extra kg of pup mass and an increase in pup mass from 10kg to 20kg results in an increase in odds of obtaining a positive PCR result from nasal swabs of 3.91.

Table 6-6 Multivariate logistic regression analysis for PhHV1 PCR of nasal swabs in live free-ranging grey seal pups. S.E. of coef: Standard error of coefficient; OR: odds ratio; 95% CI: 95% confidence interval.

Variable		Coefficient.	S.E. of coef.	Odds ratio (OR)	95% CI of OR	p-value
Intercept		-2.89	0.92	-	-	-
Mass	Mass in kg	0.17	0.017	1.15	1.08,1.22	<0.001
Site	Tidal boulder beach	-	-	1	-	-
	Rocky pools	-1.75	0.72	0.55	0.18, 1.62	0.015
	Muddy grassy slope	-2.22	0.71	0.24	0.08, 0.72	0.002

When considering all free-ranging pups (whether dead or live), stepwise logistic regression analysis indicated that, among the variables examined, only the mass was a statistically significant multivariate predictor of positive PCR result from nasal swabs (coefficient=0.07943; $P < 0.001$; odds ratio, 1.08; 95% CI: 1.04, 1.13). The interpretation is that, for each additional kg of mass the expected odds of having a positive PCR result for PhHV1 on nasal swab are 1.08.

When considering stranded live grey seal pups along with all free-ranging pups (dead and live), stepwise logistic regression analysis indicated that, among the variables examined, only the mass was a statistically significant multivariate predictor of positive PCR result from nasal

swabs (coefficient= 0.06794; $P < 0.001$; odds ratio, 1.07; 95% CI: 1.03, 1.11). However, the effect of colony (stranded versus free-ranging animals) approached statistical significance ($p=0.08218$) and was therefore included in the final logistic regression presented in Table 6-7.

Table 6-7 Multivariate logistic regression analysis for PhHV1 PCR of nasal swabs in live and dead free-ranging and live stranded grey seal pups. S.E. of coef.: Standard error of coefficient; OR: odds ratio; 95% CI: 95% confidence interval.

Variable		Coefficient.	S.E. of coef.	Odds ratio (OR)	95% CI of OR	P-value
Intercept		-1.51	0.477			
Mass	Mass in kg	0.077	0.019	1.07	1.03,1.11	0.002
Colony	Free-ranging pups	-	-	1	-	-
	Stranded pups	0.777	0.447	2.18	0.91, 5.23	0.08

When considering all live pups (stranded and free-ranging), stepwise logistic regression analysis indicated that, among the variables examined, mass and colony (stranded versus free-ranging animals) were statistically significant multivariate predictors of positive PCR result from nasal swabs. The final logistic regression for PhHV1 nasal swab PCR status is presented in Table 6-8.

Table 6-8 Multivariate logistic regression analysis for PhHV1 PCR of nasal swabs in live free-ranging and stranded grey seal pups

Variable		Coef.	S.E. of coef.	Odds ratio (OR)	95% CI of OR	P-value
Intercept		-3.107	0.797			
Mass	Mass in kg	0.128	0.03	1.09	1.04,1.14	<0.001
Colony	Free-ranging pups	-	-	1	-	-
	Stranded pups	1.592	0.55	1.2	0.51, 2.82	0.003

No correlation of PhHV-1 with mass, length or girth was seen in yearling samples.

- **Tissue samples**

The prevalence of PhHV1 DNA in pooled tissue samples was similar to that in nasal swabs (see Table 6-4). No significant difference was noted between the prevalence of PhHV1 in tissues of dead free-ranging pups when compared to dead stranded pups. In several cases only the nasal swab or the pooled tissue sample was positive for PhHV1. The presence of PhHV1 DNA in nasal swabs was strongly correlated with the presence of PhHV1 DNA in pooled tissue samples ($p=0.005$).

In order to investigate the relationship between PhHV1 and the presence of specific lesions in dead pups, pups with a positive result in either of the two tests (nasal swab or the pooled tissue sample) were considered as having a positive PhHV1 status.

In dead pups, a positive PhHV1 PCR result was significantly related to the presence of hepatic necrosis, thymic atrophy and buccal ulceration (see Table 6-9). There was, however, no relationship between PhHV1 infection and interstitial pneumonia or encephalitis.

Table 6-9 Odds ratio of finding lesions in stranded dead and free-ranging dead pups with a positive PhHV1 PCR status. (OR: odds ratio; 95% CI: 95% confidence interval; Inf: Infinity; Sign: Statistical significance of results; NS: non-significant; *: p<0.05; **: p<0.01; ***: p<0.001)

Lesion	PhHV-1 status	number (%) positive	OR	95% CI	P-value	Sign.
Adrenal necrosis	HV -	1 (5.9%)	3.20	0.36, 28.23	0.29	NS
	HV +	7 (16.7%)				
Hepatic necrosis	HV -	1 (5.9%)	16.00	1.93, 132.39	0.01	*
	HV +	20 (50%)				
Multifocal hepatitis	HV -	4 (23.5%)	2.00	0.55, 7.21	0.289	NS
	HV +	16 (38.1%)				
Thymic Atrophy	HV -	4 (23.5%)	14.39	3.59, 57.71	<0.001	***
	HV +	31 (81.6%)				
Buccal ulceration	HV -	1 (5.9%)	10.88	1.32, 89.93	0.027	*
	HV +	17 (40.5%)				
Tongue ulceration	HV -	3 (17.6%)	1.66	0.4, 6.88	0.488	NS
	HV +	11 (26.2%)				
Keratitis	HV -	0 (0%)	33091159	0, Inf	0.995	NS
	HV +	4 (9.5%)				
Uveitis	HV -	0 (0%)	44909430	0, Inf	0.995	NS
	HV +	5 (12.5%)				
Interstitial pneumonia	HV -	7 (41.2%)	1.57	0.5,4.91	0.437	NS
	HV +	22 (52.4%)				

Pups presenting with mild or moderate to severe thymic atrophy were significantly more likely to be PhHV1 positive than pups with minimal or no evidence of thymic atrophy (FET, p<0.001) (Table 6-9). The degree of thymic atrophy was significantly related to the PhHV1 status of the pups examined (Figure 6-2).

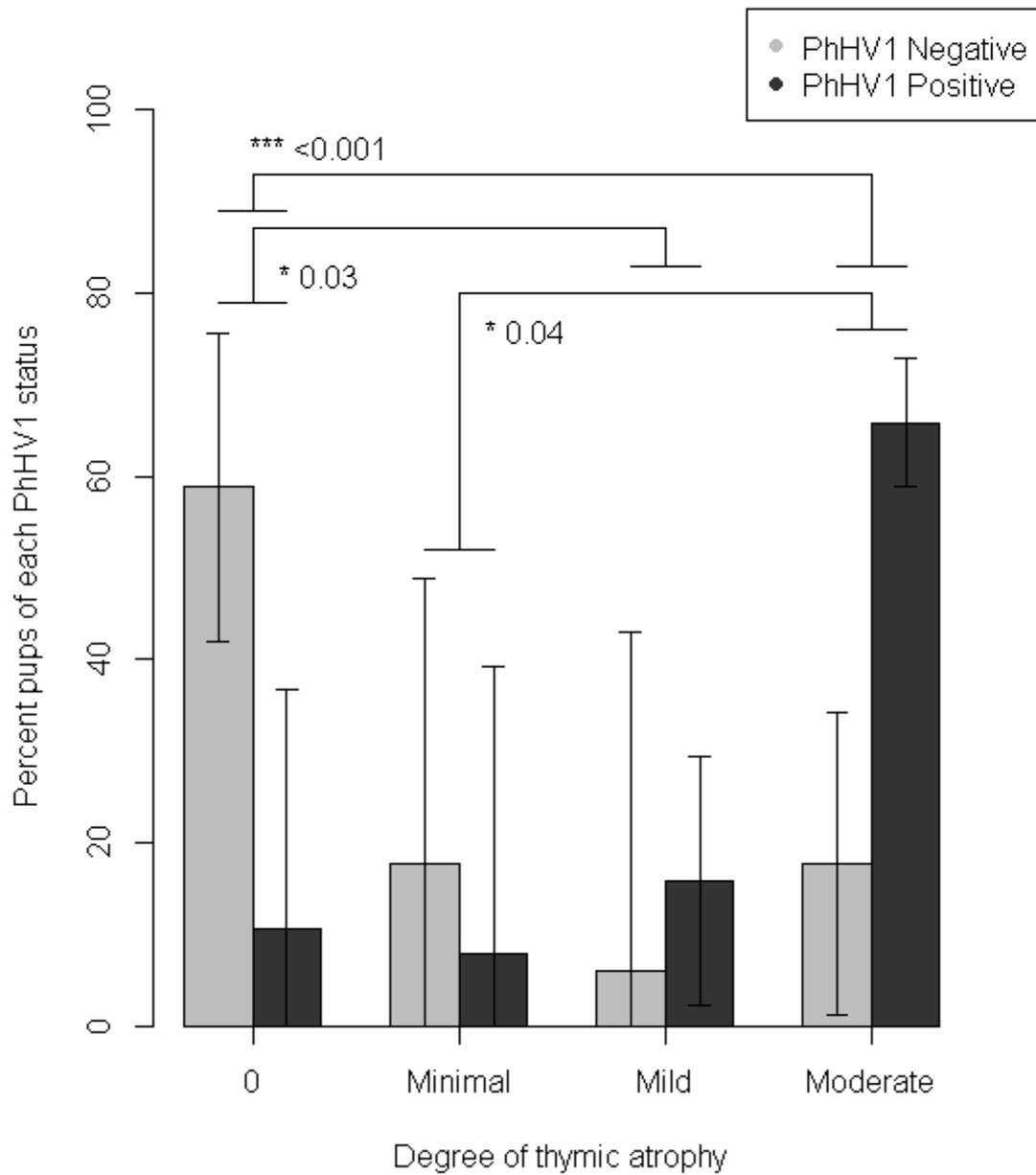


Figure 6-2 PhHV1 status of stranded dead and free-ranging dead grey seal pups presenting with differing degrees of thymic atrophy; Bars represent 95% confidence interval; Significance brackets represent result of generalised linear model comparing prevalence of PhHV1 within groups of pups presenting each degree of thymic atrophy

In addition, the presence of thymic atrophy is positively correlated with the cortical to medullary ratio in the adrenal glands, an indicator of stress, with a coefficient of 1.4689 and $p=0.006$.

- **Immunohistochemistry**

Immunohistochemistry using antibodies targeting both Bovid HV1 and Felid HV1, to evaluate possible cross-reaction with PhHV1, was unrewarding. Appropriate positive control tissues showed clear immunolabelling for both Bovid HV1 and Felid HV1 indicating that the specific IHC was valid.

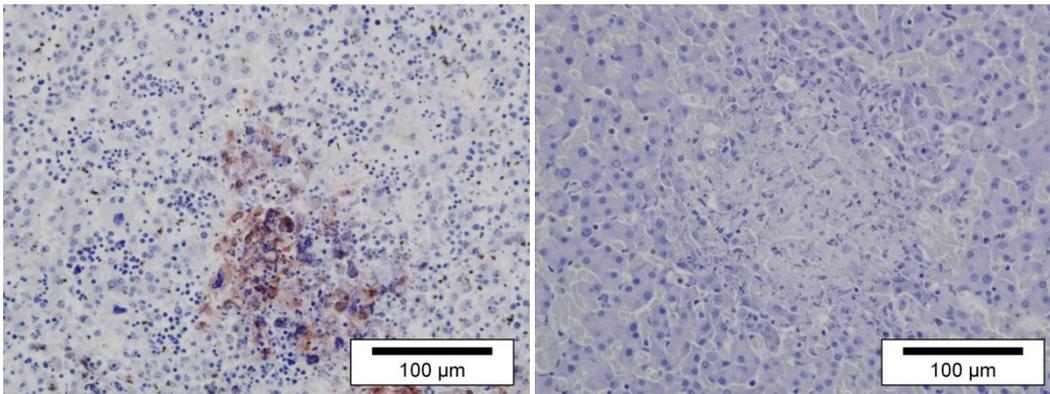


Figure 6-3 Immunohistochemical labelling of Bovid Herpes virus 1. A: Control tissue (bovine liver experimentally infected with BoHV1). Note dark brown immunolabelling within a focus of hepatic necrosis and inflammation. B: Test tissue. Seal liver with hepatic necrosis. Absence of labelling. IHC.

6.3.3 Phocid Herpesvirus 2

PhHV2 DNA was not detected in any of the pooled tissue samples from dead pups on the colony or at the rehabilitation centre.

Bands of 430bp molecular weight were identified in DNA extracted from the buffy coat of four live yearlings (Y005, Y008, Y010 and Y017) as well as in positive control lanes. Multiple faint bands of variable molecular weight were also seen either side of the 430bp band (see Figure 6-4). Very faint bands of variable molecular weight, deemed non-specific PCR product were also noted in several other lanes.

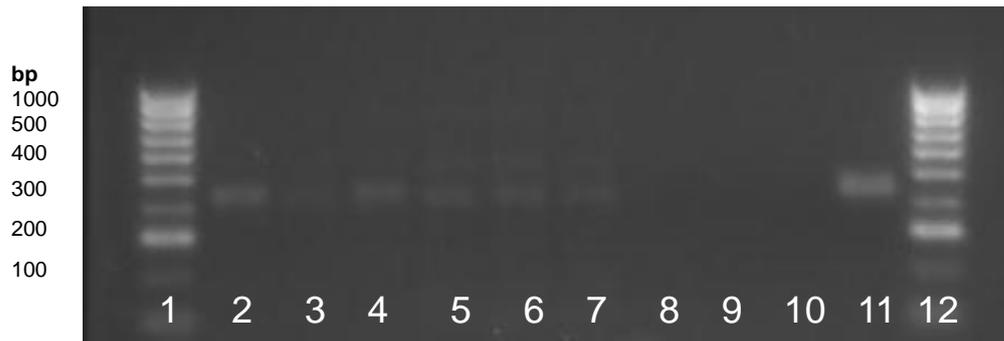


Figure 6-4 Gel image showing 430bp PhHV-2 UL52 PCR product. Lanes 1 and 12 (from left), Bioline Hyperladder IV marker (100bp); Lanes 2-7: Yearling samples (Y005, Y008, Y010, Y017, Y0017, Y017, respectively); Lane 8: No Template control (NTC); Lanes 9 and 10: Extraction controls (ExC A and ExC B); Lane 11: Positive control (Herbie)

- **Sequence analyses of UL52 amplicons of different PhHV-2 isolates**

Amplified DNA from all samples showing distinct bands (samples Y005, Y008, Y010, Y017) and the positive control sample (Herbie) were sequenced. The sequencing reaction of the amplicon from Y008 was not interpretable based on the chromatogram results following sequencing (Figure 6-5). The PCR result was therefore deemed inconclusive and this sample was excluded from further analysis.



Figure 6-5 Chromatogram of sequence reads of positive control sample (A) and test samples Y005 (B) and Y008 (C), showing clear peaks for Y005 and overlapping reads in the case of Y008

- **Phylogeny of PhHV2 UL52 gene sequences**

Nucleotide BLAST® (Basic Local Alignment Search Tool, NCBI) of the sequence obtained from amplicons detected in samples Y005, Y010 and Y017 showed 99% homology to the UL52' helicase-primase complex homolog isolated from phocid herpesvirus type-2 PhHV-2 of *Phoca vitulina*, isolate 7848 (GenBank Accession number S81230).

Two phylogenetic clusters of viruses were distinguishable (Figure 6-6): One cluster contained the three Scottish isolates from samples Y005, Y010 and Y017 and a second cluster contained the four Dutch isolates published by Martina *et al.* in 2003 along with the positive control sample, Herbie (the latter appearing in figure 1 of the aforementioned publication).

These results imply that a minimum of 3 samples of the 19 yearling buffy coats tested were positive for PhHV-2 based on sequencing of the PCR product. It is difficult to assess the status of the remaining yearling buffy coat samples without sequencing the PCR product of all

samples. A minimum prevalence of 15.8% can be established in yearlings based on these results. Time constraints precluded further work on this agent.

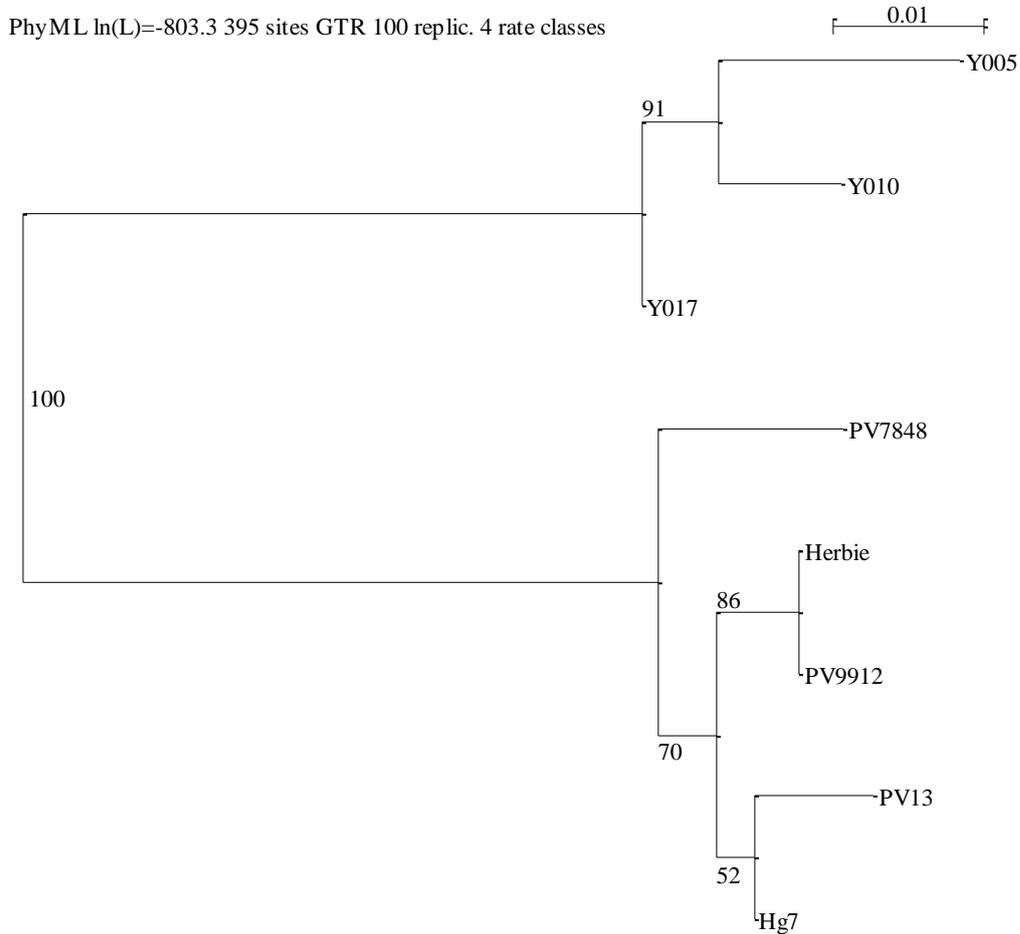


Figure 6-6 Phylogenetic tree of aligned consensus reads of three yearling samples, positive control sample and 4 published sequences of UL52 (Martina et al. 2003), generated using Seaview software using the fast maximum likelihood program phyML, with 100 bootstrap replicates

6.3.4 Morbillivirus PCR

Phocine distemper viral RNA was not detected in any of the pooled tissue samples from dead free-ranging pups or dead stranded pups. As there was no reported PDV outbreak or disease at the time of sampling this result was not unexpected.

6.3.5 Poxvirus PCR

Parapoxvirus DNA was detected in only one of the pooled tissue samples from stranded dead pups and in no pooled tissue samples of free-ranging dead pups. Pup R005, presented with multiple raised skin lesions on the ventral aspect of its fore flippers, characteristic of seal pox lesions, and was positive on qPCR for the virus with an average Ct value of 35.2 cycles.

The distribution of parapoxviral DNA within (11) individual tissues of pup R005 detected poxviral DNA only in 3 tissues: skin, lungs and bronchial lymph node. Tonsil, mesenteric lymph node, ileo-caeco-colic junction, showed inconsistent amplification of parapoxvirus with Ct values of 43 and above and were considered weekly positive. . Pooled tissue samples of pup R006 showed very late amplification in 2 of 3 replicates.

6.3.6 *Toxoplasma gondii* and *Neospora caninum* PCR

A single replicate from each of three tissue pools of tongue, lung, brain stem and heart tissue were positive for *Toxoplasma gondii* on PCR (pups CD008, CD038 and CD043). Subsequent PCR assays carried out on individual tissues were negative. *Toxoplasma* spp. was not identified on any of the sections processed for immunohistochemistry. Table 6-10 presents the main characteristics and pathological findings in these three pups.

None of the pooled tissue samples were positive on PCR for *Neospora caninum*.

Table 6-10 Signalment, weight and main pathological findings in three pups with positive *T. gondii* PCR results on pooled tissue samples

Seal	Sex	Stage	Weight (kg)	Pathology
CD008	Female	I	11.6	Starvation; septicaemia due to <i>Streptococcus dysgalactiae</i>
CD038	Male	IV	20.8	Septicaemia (<i>Pseudomonas aeruginosa</i>); meningo-encephalitis and terminal aspiration pneumonia
CD043	Female	I	14.2	Omphalitis-peritonitis-hepatitis complex with severe hepatic necrosis, fibrinous bronchopneumonia (<i>E. coli</i> in septicaemic distribution)

6.3.7 Occurrence of multiple pathogens in individual animal

Previous chapters have considered individual epidemiology of each pathogen. Given that pathogens could be considered as additional environmental stressors, the interaction between these agents was investigated.

- **Co-infection of *Salmonella* spp., *Campylobacter* spp. and PhHV1**

Co-infection of live free-ranging grey seal pups with *Campylobacter*, *Salmonella* and PhHV1 is illustrated in Figure 6-7. No correlation was noted between the prevalence of *Campylobacter* and *Salmonella* in live free-ranging grey seal pups ($p=0.17$, FET) or between the prevalence of *Campylobacter* and PhHV1 ($p= 0.53$, FET).

The prevalence of *Salmonella* spp. and PhHV1 showed a statistically significant association ($p=0.011$, FET). This association was therefore investigated further using multivariate logistic regression (glm) with binomial errors with isolation of *Salmonella* as the outcome variable and detection of PhHV1, pup mass and site as fixed variables. The effect of PhHV1 disappeared using stepwise analysis when site and mass were included this model. Indeed, pup mass being strongly correlated with both PhHV1 and *Salmonella*, the relationship, if any, between these two agents could not be determined due to this strong confounding factor.

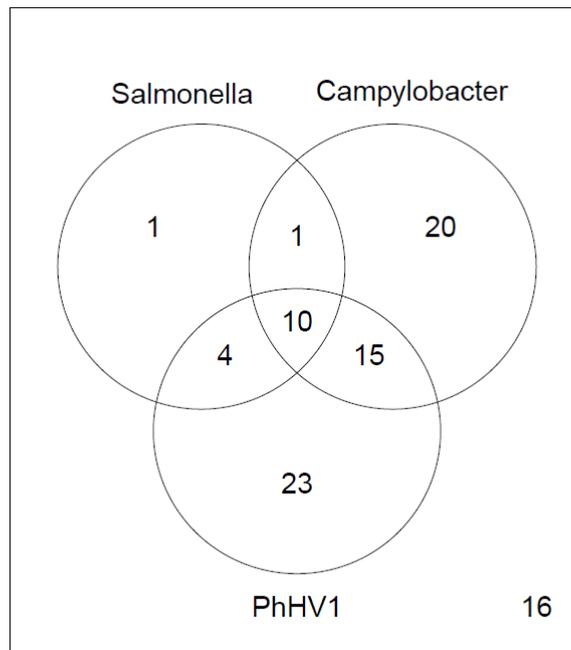


Figure 6-7 Venn diagram showing number of live free-ranging grey seal pups (n=90) in which either *Salmonella* spp., *Campylobacter* spp. or PhHV1 was detected. Numbers within overlapping portions of the circles represent numbers of animals co-infected with two or more of these agents.

- **Co-infection of *Listeria* spp. and phocid herpesvirus 1**

In Chapter 3, the presence of *L. monocytogenes* was shown to be significantly higher in pups with mild to severe thymic atrophy and higher, although not statistically significantly so, in pups with hepatic necrosis. In parallel, a strong correlation of both mild to severe thymic atrophy and hepatic necrosis with the presence of PhHV1 was demonstrated in the previous paragraphs. No correlation was found between the presence of these two pathogens (FET, $p=0.51$).

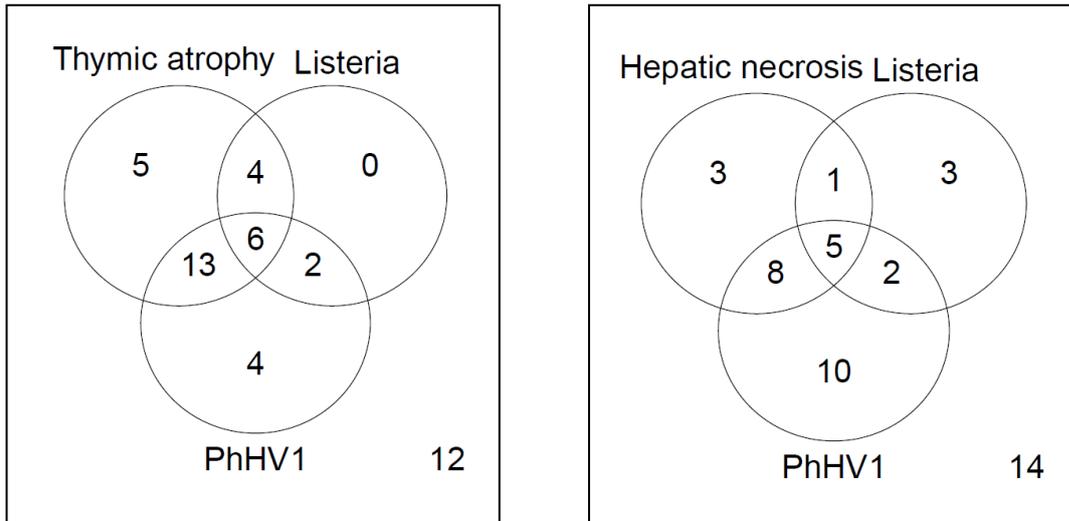


Figure 6-8 Venn diagrams showing number of dead free-ranging grey seal pups in which either thymic atrophy or hepatic necrosis was noted, along with the presence of *Listeria* spp. and / or nasal shedding of PhHV1 or co-infections of these agents

GLM model building considered the presence of each lesion (present vs absent) as the outcome variable and included the two pathogens: *Listeria* and PhHV1 and mass as covariates.

The best fitting model for thymic atrophy included pup mass and nasal shedding of PhHV1 as the main explanatory factors. Thymic atrophy was therefore correlated with nasal shedding of PhHV1 and, in the absence of nasal shedding of PhHV1, a lower pup mass.

Table 6-11 Multivariate logistic regression analysis showing the factors associated with risk of finding mild to marked thymic atrophy in dead free-ranging grey seal pups. S.E.: standard error of coefficient; 95% CI: 95% confidence interval; Sign: Statistical significance of results; NS: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; . = $p < 0.1$

Variable	Coefficient	S.E.	Odds ratio (OR)	95% CI of OR	P-value	Sign.
Intercept	3.10	1.28				
PhHV1						
Negative	-	-	1	-	-	
Positive	12.82	1.01	4.22	1.2, 14.9	0.016	*
Pup Mass	-0.24	0.09	0.88	0.79, 0.99	0.006	**

Stepwise logistic regression investigating relationships with the presence of hepatic necrosis, showed the presence of *Listeria* to be non-significant when nasal shedding of PhHV1 and pup mass were included in the model. The final model predicting the presence of hepatic necrosis showed both nasal shedding of PhHV1 and pup mass to be statistically significant. This implies that the likelihood of having thymic atrophy or hepatic necrosis is higher in pups with nasal shedding of PhHV1 and, in the absence of nasal shedding of PhHV1, these lesions are more frequent in pups with a lower mass.

Table 6-12 Multivariate logistic regression analysis showing the factors associated with risk of finding hepatic necrosis in dead free-ranging grey seal pups. S.E.: standard error of coefficient; 95% CI: 95% confidence interval; Sign: Statistical significance of results; NS: not significant; *: p<0.05; **: p<0.01; ***: p<0.001; . = p<0.1

Variable	Coefficient	S.E.	Odds ratio (OR)	95% CI of OR	P-value	Sign.
Intercept	-0.37	1.04				
PhHV1						
Negative	-	-	1	-	-	
Positive	1.99	0.76	4.6	1.2, 17.63	0.009	**
Pup Mass	-0.13	0.07	0.92	0.82, 1.03	0.05	*

6.4 Discussion

6.4.1 Phocid herpesvirus 1

A high prevalence of PhHV1 was found in nasal swabs of all study groups of grey seals (57.8% to 88.9% in pups). The prevalence was highest in dead, stranded animals (88.9% although given the small size of this particular group size (n = 9), the true significance of this finding for the population can only be inferred. In particular, comparison of the PhHV1 status of stranded live grey seal pups on arrival compared to their PhHV1 status on arrival at the rehabilitation centre and at time of post-mortem examination showed an increased prevalence of nasal shedding of PhHV1, which may be due to stress-induced viral replication or maybe imply transmission of virus within individuals at the rehabilitation centre, however this difference is not significant.

Goldstein *et al.* (2004) demonstrated that a similarly high proportion (40%) of free-ranging pre- and post-weaned harbour seal pups were PCR positive for PhHV1 DNA compared to only 3% of harbour seals over 1 year of age on nasal swabs.

Martina *et al.* (2002) suggested that juvenile harbour seals and harbour seal pups are more prone to the development of severe PhHV1 infections than either adult harbour seals or grey seal pups. These authors were not able to compare the severity of clinical infection in different age groups of grey seals but did describe “severe disease” including a mucopurulent nasal and ocular discharges, epistaxis and severe dyspnoea, occasionally accompanied by vomiting and diarrhoea, with rectal temperatures of over 38.5°C in 7% of grey seal pups.

On the Isle of May breeding colony during 2011 there was an increase in the number of pups shedding PhHV1 over time (as the breeding season progressed), in parallel with the increased pup mass as animals reached the end of the lactation period. A real time PCR assay targeting an 115bp sequence of the PhHV1 glycoprotein B (gB) gene was published in 2013 (Roth *et al.* 2013). Future work, using this newly published method, could aim to quantify the amount of PhHV1 shed in nasal swabs using the newly published PCR method, and maybe lead to a better understanding of the identification of the source (reservoir) of the initial infection for pups born at the beginning of the breeding season (yearling vs. dams, see below) and also the potential of virus transmission between pups.

PhHV1 DNA was detected in nasal swabs from 27.8% of yearlings. Given the high prevalence of PhHV1 detected in pups on the colony, it is unlikely that this represents a first infection of these yearlings with PhHV1. It is more likely to represent re-activation of a latent PhHV1 infection. Post-weaning to yearling grey seals undergo dramatic physiological changes throughout their first year including a switch in body composition from 13% to 20% protein at the expense of fat, which decreases from 40% to 12% body weight (Hall & McConnell 2007). A plausible hypothesis may be that there is some form of energetic trade-off between resources for

immunity and protein deposition, possibly compounded by the stress of returning to the breeding colony, that leads to this viral reactivation.

Given the nature of the study, it was not possible to assess tissue carriage of PhHV1 in live pups. However, the number of pups infected with PhHV1 is likely to be substantially higher than the number of pups shedding the virus in nasal secretions. Given that the estimated prevalence in live free-ranging pups was 57.8%, it is plausible that a large majority of pups born on this colony would have been infected by the virus by the end of the pupping season. As a crude measure, if one assumes that stage II and stage V pups are of sufficiently different ages (average 4 days vs 18 days) and that the duration of PhHV1 nasal shedding does not exceed 14 days (7-19 days according to Goldstein *et al.* (2004), shedding in stage V pups is likely to represent newly infected pups. The combined prevalence of PhHV1 in stage II (39%) and stage V pups (66.7%) measured in this study would support a very high risk of exposure of pups to the virus. Such a high prevalence would be typical of host-adapted alpha herpesviruses in densely populated breeding colonies, such as is seen in breeding catteries with feline herpesvirus 1 (FeHV1) and breeding kennels with canine herpesvirus 1 (CaHV1) (Gaskell *et al.* 2007; Gaskell & Willoughby 1999). Serology of older pups may have been able to confirm this hypothesis and serological assessment of sub-adult or adult animals may also be beneficial to determine PhHV1 exposure rates and disease significance. If the average duration of nasal secretion of PhHV1 in grey seal pups were known, the cumulative exposure of pups to PhHV1 could be simulated.

The most significant factor correlating with PhHV1 detection based on our model was increased pup mass, which was itself confounded with age. None of the stillborn pups were positive for PhHV1, suggesting that trans-placental infection does not occur. Animals infected with alpha herpesviruses, such as canine herpesvirus 1 and feline herpesvirus 1, tend to become

persistently infected, with the virus typically becoming latent in tissues, such as the trigeminal ganglion, and recrudescing in times of stress. Transmission often occurs at or around parturition, with recrudescence of the virus in maternal tissues and transmission to the neonate via vaginal secretions or nasal shedding (Gaskell & Willoughby 1999; Schlafer & Miller 2007). Goldstein *et al.* (2004) showed that viral shedding in nasal secretions of stranded harbour seal pups occurred 4–7 days post direct contact exposure. These findings could explain the low prevalence of nasal shedding in early stage pups in the present study. Indeed, a negative PCR result in nasal swabs in early stage pups would not rule out the possibility of peri-natal transmission within mother-pup pairs. In addition, maternally derived antibodies may contribute to initial protection. However, as maternal antibodies decline, susceptibility to infection increases.

The higher prevalence of PhHV1 among live free-ranging pups on the tidal boulder beach site may be a consequence of regular displacement and subsequent crowding of these pups due to the twice daily high tides. This may lead to higher stress levels and increased contact between pups and other adult seals. In contrast, the rocky pool site is the most secluded of the 3 sites, which may result in less disruption and movement and therefore less contact between pups and non-parental adults.

In dead pups, a positive PhHV1 PCR status significantly correlated with hepatic necrosis, thymic atrophy and buccal ulceration. In domestic dogs, canid herpesvirus 1 (CaHV1) infection can cause an acute, severe and often fatal disease of neonates (less than 3 weeks of age) with endotheliotropism of the virus leading to serosal haemorrhages and characteristic petechial haemorrhage over the adrenal and renal surfaces. Other lesions in canine neonates include 1) focal necrosis with intralésional intranuclear inclusion bodies in liver and other parenchymal organs, 2) non suppurative meningoencephalitis, occasionally associated with areas of necrosis within the cerebral cortex and 3) vascular endothelial hypertrophy and hyperplasia associated

with mononuclear infiltrates (Maxie and Youssef, 2007). In older puppies, around 12 weeks of age, CaHV1 has been shown to cause necrotising rhinitis, bronchointerstitial pneumonia and multifocal alveolar necrosis and the virus has been associated with adult dogs presenting with tracheobronchitis (kennel cough) (Caswell and Williams 2007). CaHV1 has different tissue tropisms depending on the age of the animal infected with endotheliotropism in neonates but an increasing affinity for respiratory epithelium as time progresses, possibly mediated by poor thermoregulation in neonates (Maxie & Youssef 2007).

Thymic atrophy is a frequent observation in the course of infectious disease in neonates. It can be initiated by cytokines such as tumour necrosis factor α (TNF α) or due to a rise in systemic glucocorticoid hormone levels such as occurs during infection as they can trigger apoptosis in (immature) CD4+CD8+ thymocytes, by activating caspase-3, -8, and -9 (Savino 2006).

The present study demonstrated that the presence of thymic atrophy was associated with a higher cortical to medullary ratio in the adrenal gland. As glucocorticoids are produced in the zona fasciculata of the adrenal cortex, this adds support to the hypothesis that thymic atrophy may be triggered by stress and subsequent glucocorticoid hormone increase and is further supported by the demonstration, previously, of higher baseline cortisol levels in stranded harbour seal pups which survived following rehabilitation (Gulland et al. 1999).

The pathology of PhHV1 in grey seals is poorly studied and the presence or absence of the virus in different tissues would be worth investigating. Tissue samples from 13 organs of each dead pup have been prospectively stored in VTM for this purpose, but time limitations prohibited furthering this part of the study. Likewise, quantitation of virus load or transcription levels of RNA in each tissue would be useful also and the disappointing outcome of the IHC may justify

the development of an in-situ hybridisation probe to determine if virus is primarily associated with histological lesions.

This is further justified by Goldstein *et al.* (2005) who found that nasal shedding of PhHV1 was not a consistent finding in infected animals despite positive PCR results in multiple other tissues and organs of harbour seal pups (Goldstein *et al.* 2005).

The apparently similar prevalence in live and dead pups in the present study most likely reflects the ubiquitous nature of the virus, however, a real-time PCR assay may show a difference in PhHV1 load in swabs between dead and live pups, helping to elucidate the true pathogenicity of this virus and its significance to grey seal pup survival.

The presence of hepatic necrosis or thymic atrophy is correlated with PhHV1, and, in the absence of PhHV1, with a lower pup mass. This most likely reflects a proportion of pups succumbing to starvation induced immunosuppression with alternative causes of hepatic necrosis such as *E. coli* or *Listeria monocytogenes* as discussed in Chapter 3.

6.4.2 Phocid Herpesvirus 2

PCR did not detect PhHV2 in tissues of any of the pups. However, amplification of PhHV2 DNA, subsequently confirmed by sequencing, was obtained from buffy coats of 3 free-living, live grey seal yearlings, giving a minimal prevalence estimate in this group of 15.8%.

Comparison with sequences obtained from Dutch PhHV2 strains (Martina *et al.* 2003) showed a 99% homology, confirming that PhHV2 is circulating in seals in Scottish coastal waters, yet clearly separated the sequences from the three Scottish cases from the Dutch strains.

This suggests, at least for the 2011 pupping season on the Isle of May, that infection with PhHV2 occurs after the pups have departed from the breeding colony, probably during the course of the first year and that the virus can be detected by PCR within buffy coats.

It was not possible to assess any link between PhHV2 carriage and pathology from the present sample set. Martina *et al.* (2003) found no clear disease associations in seals naturally infected with PhHV2 viruses but did present circumstantial evidence to suggest that the virus might be linked to respiratory disease (Martina *et al.* 2003). These authors also described a highly cytotoxic effect of PhHV2 on peripheral blood mononuclear cells and suggested that the virus may induce an immunosuppressive effect.

The presence of multiple bands of different molecular weights on the PCR gel suggests that a degree of non-specific amplification is occurring during the PCR reaction. This implies low specificity of the assay, explaining the need to sequence the PCR product in 4 cases, and highlights also the need to develop a more specific and reliable assay for this pathogen.

Development of a real-time PCR assay targeting PhHV2 would be a welcome addition to the diagnostic tool-kit available for pinnipeds. Given an improved PhHV2 assay, it would be useful to determine its prevalence in a much larger group of yearlings; ideally carrying out a longitudinal cohort study, sampling pups immediately prior to entering the sea and at specific time points during their first year of life.

6.4.3 Morbillivirus

The absence of phocine distemper virus (PDV) RNA in any of the samples tested was an anticipated outcome of this study. PDV in Scottish grey seal populations has historically only been seen in parallel with large scale mortalities in the sympatric harbour seal. Although grey seals were initially highlighted as asymptomatic carriers during an outbreak, their potential to act as a reservoir for the virus between outbreaks still needs to be investigated (Hammond *et al.* 2005b). Several serological studies of grey and harbour seals suggest that PDV is not endemic in Scottish seal populations, but rather is introduced sporadically and that grey seals may

represent a vector of this disease into harbour seal populations rather than a reservoir (Pomeroy et al. 2005). The results of our study add further evidence for the lack of PDV in Scottish grey seals.

6.4.4 Poxvirus

The only pup in which poxvirus DNA was unequivocally detected was in the stranded, dead pup (R005) which presented with large, proliferative skin lesions consistent with poxvirus infection. The distribution of poxvirus within 11 different organs unequivocally detected poxvirus DNA in only three tissues (skin, lung and bronchial lymph node). Parapoxvirus infection tends to be restricted to the skin in the large majority of mammalian species (Ginn et al. 2007), so the presence of viral DNA in lung and bronchial lymph node samples may reflect inhalation of virus particles during grooming or feeding rather than a tropism for the respiratory system. This pup had severe thymic atrophy, concurrent PhHV1 infection, *Pseudomonas aeruginosa* infection, jaundice and perivascular cuffing and gliosis in the brain. Clinical progression, manifested by poxviral dermatitis, may have been permitted by severe immunosuppression, reflected by the severe thymic atrophy and high adrenal cortical to medullary ratio. However, the underlying cause of this immunosuppression was not definitively diagnosed.

Pup R006 showed inconsistent and weak amplification of poxviral DNA in the pooled tissue sample. However, the post mortem examination of pup R006 was performed within 2 hours of that of pup R005, in the same post-mortem suite. Given the lack of skin lesions, inconsistency of DNA amplification and the presence of very low copy numbers of poxviral DNA in this sample, it could reflect contamination of tissues at the time of post mortem examination. Although, given that both pups were cohabiting at the rehabilitation centre it is not possible to fully exclude the possibility that pup R006 had a subclinical infection with poxvirus or was acting as a fomite if they were cohabiting.

In the large majority of terrestrial and aquatic animal species, parapoxvirus infection is self-limiting, despite inducing proliferation, vacuolation and ultimately degeneration of keratinocytes. Grossly, infection manifests as multiple erythematous, to papular to proliferative growths, distinct from the more vesicular and pustular nature of lesions induced by Orthopoxviridae, which can progress to a systemic distribution, unlike Parapoxviridae (Ginn et al. 2007). Parapoxvirus infection is not known to be fatal in pinnipeds but can interfere with feeding (Roess et al. 2011). Lesions have been described in the mouth, flippers and ventrum of harbour seals (Muller et al. 2003), whereas poxviral infection results in lesions predominantly located on the head and neck in California sea lions (*Zalophus californianus*) (Nollens et al. 2006). It is thought that the parapoxvirus enters the host via small breaches in the integument and that the location of the resulting lesions is influenced by husbandry practices (such as tube feeding) or the abrasiveness of the environment (substrate) that pups encounter (Hicks & Worthy 1987).

In harbour seals, the incubation period has been estimated to be 3 weeks (Muller et al. 2003). Muller *et al.* (2003) suggested that grey seals may act as carriers of poxvirus infection to the Northern part of the German Wadden Sea where they demonstrated substantial outbreaks of poxvirus in rehabilitating harbour seals. The lack of maternal immunoglobulin transfer may have accounted for the outbreak in the 1 to 10 day old German harbour seals.

6.4.5 *Toxoplasma gondii* and *Neospora caninum*

Finding *Toxoplasma* spp. DNA in pooled tissue samples of free-ranging grey seal pups is noteworthy. The pathogenicity of this agent is unclear as no lesions could be unequivocally attributed to this pathogen as the *T. gondii* antigen was not detected by IHC and so could not be co-localised with histological lesions. The negative outcome of IHC in the present study is not surprising given that detection of *T. gondii* cysts in grey seal tissues, even in experimental

infection, is very rare. Indeed, only a single tissue cyst was detected in the study carried out by Gajadhar *et al.* (2004) which showed that negative histological (H&E stain and immunohistochemistry) results cannot be relied on for ruling out *T. gondii* as a cause of disease in grey seals. The pups in the present study probably succumbed to other disease conditions before *T. gondii* infection resulted in significant lesions. This is supported by the presence of *T. gondii* DNA in only one of three replicates of each pooled tissue sample and the complete lack of amplification from individual tissues and suggests that the burden of *T. gondii* was very low. It was not possible to determine whether the *T. gondii* tachyzoites were present within the seal tissues or present on their surface given that tongue samples were included in the pooled tissue sample and that pup CD038 had terminal aspiration pneumonia leaving sediment within lung tissues. Nevertheless, the results imply that grey seal pups are exposed to *T. gondii* on the Isle of May.

European grey seals are known to become exposed to *T. gondii*, with a seroprevalence estimated at 23.4% in a sample of live, free-ranging grey seal pups, juveniles and adults (Cabezón *et al.* 2011). However the source of the exposure was not determined. In the case of the Isle of May, there had been no resident cats on the island for 25 years prior to the study (Ailsa Hall, SMRU, pers. comm.). Given that domestic and wild felids are the only known definitive hosts for *T. gondii* and that survival of oocysts in sea water has been shown to exceed 2 years (Lindsay & Dubey 2009), the presence of *Toxoplasma* in marine species implicates land-based surface runoff into the sea as the source of infection for marine mammals (Miller *et al.* 2002). Therefore most likely sources for animals in the present study were coastal run-off of *T. gondii* from domestic cats or maternal vertical transmission to grey seal pups. Pathways of *T. gondii* transmission throughout the marine environment are poorly understood but filter-feeding fish such as northern anchovies (*Engraulis mordax*) and Pacific sardines (*Sardinops sagax*) have been shown experimentally to be a plausible vector for piscivorous marine mammals such as seals (Massie *et al.* 2010).

Neospora caninum DNA was not detected by PCR in any of the 59 dead grey seal pups. The only known definitive host of *Neospora caninum* is the domestic dog (*Canis lupus familiaris*) (Basso et al. 2001), so the presence of this pathogen in the marine environment would suggest contamination from terrestrial sources (Dubey et al. 2006). To date there is only serological evidence of natural exposure of *N. caninum* in marine mammals (Dubey et al. 2006; Fujii et al. 2007) whilst its pathogenicity and true prevalence remains to be investigated.

6.5 Conclusion

By applying a systematic surveillance approach to live and dead, free-ranging and stranded, grey seal pups and yearlings, a baseline prevalence of known grey seal pathogens was established for these study groups. Having such a baseline will enable better interpretation of variations across geographical locations and time. A longer term study, repeating this approach over several consecutive years would significantly add to the value of this data.

With the exception of phocine distemper virus, all pathogens investigated were present in one or more of the study groups. Phocid herpesvirus 1 was widespread in all study groups. The presence of *T. gondii* demonstrates that grey seals may act as sentinels for environmental accumulation of zoonotic pathogens, probably due to discharge of sewerage or surface water runoff.

The main advantage of this study was in establishing the presence of multiple pathogens in the same individual animals which also enabled comparison of pups of same or similar age in both free-ranging and stranded scenarios. Many previous studies, due to logistical constraints measured the presence of different pathogens in very different groups of animals (Greig et al. 2010), hindering comparisons between groups of similar ages or disease status. Furthermore, this approach makes it feasible to study the effect of multiple pathogens in one same host.

The list of pathogens selected for this study was far from exhaustive. If time (and access to positive control material) had allowed, screening for additional viruses would have been performed, in particular marine Caliciviridae, Influenza virus and Orthopoxviridae. Influenza virus and Orthopoxviridae have both been reported in grey seals (Fouchier et al. 2001; Osterhaus et al. 2000) but the prevalence of both these viruses, along with the pathogenicity of Orthopoxviridae, are poorly understood. Caliciviruses are a significant cause of disease in pinnipeds worldwide, largely represented by the San Miguel Sea Lion virus (SMSV). A mixed poxvirus and calicivirus infection was described in the skin and lips of two grey seals in 1993 (Stack et al. 1993). Lesions were typical of parapoxviral infections but there was no clear evidence that the calicivirus was playing a pathogenic role in this case. However, the widespread presence of shallow, bilateral ulcers in grey seal pups is reminiscent of the lesions seen in feline calicivirus infection and would warrant further investigation.

Chapter 7 : General Discussion: Disease and pathogen carriage in Scottish grey seals

This cross-sectional pathological study of disease in neonates on a grey seal breeding colony, Isle of May, Scotland, utilised detailed systematic post-mortem examinations, on-site bacteriology, optimal sample storage and advanced genetic techniques to investigate the causes of neonatal death. The work presented here highlights two key findings: firstly, despite significant changes in environmental pressures and population dynamics since the 1980s, the causes of death of grey seal pups on the Isle of May colony have not significantly altered; secondly, it demonstrates that grey seal pups are infected with two key genera of zoonotic bacteria and a terrestrial protozoan parasite indicating involvement of terrestrial mammalian pathogens in marine mammal disease and a role for these animals as “sentinels” of coastal microbial marine pollution.

7.1 Neonatal mortality in grey seals

This study intentionally, rigorously replicated the study of neonatal mortality carried out in 1986 on this same colony (Baker & Baker 1988) whilst utilising the significant advances both in molecular techniques and sample preservation. In particular, this study achieved higher quality bacteriology due to rapid on-site processing of samples and was also able to investigate viral and protozoal pathogens due to the combination of optimal sample storage and molecular techniques.

The grey seal population on the Isle of May has grown exponentially since the 1970s and is currently levelling off, most likely through the effects of density dependence. In 1986, at the time of the previous study of neonatal mortality, the colony was undergoing the exponential growth phase with approximately 1000 pups born annually, whereas in 2011, at the time of the present study, with 2300 pups born annually the population was entering the stationary phase of the logistic growth curve. This thesis investigated the contribution of neonatal mortality to the slowing of population growth. Despite the significant increase in pup production over the last 25 years, the causes of death of grey seal pups on the Isle of May colony have not significantly changed with the principal causes of death being starvation (30%); omphalitis-peritonitis (26%), septicaemia (22%), stillbirth (10%) and trauma (4%). This suggests that neonatal mortality is unlikely to be one of the density dependent factors driving the levelling off of population growth on this colony. Although detailed studies of mortality counts are scarce, recent ground counts of 12.4% and 9.9% in 2008 and 2012 respectively (Chris Morris, SMRU, pers. comm.) are comparable to those obtained by Baker in 1986 (12.5%) (Baker & Baker 1988). This observation further supports the above hypothesis that neonatal mortality is unlikely to be playing a significant role in the population dynamics of this colony.

In the absence of increasing neonatal mortality or changes in cause of neonatal mortality, factors such as juvenile survival, adult survival, emigration or fertility may be driving the levelling off of the Isle of May grey seal population. Delayed recruitment of females into the breeding population has indeed been described in this population (Pomeroy et al. 2010). In addition, despite their strong philopatry, migration of grey seals from the Isle of May to breed on newly founded neighbouring colonies, such as Fast Castle, has been suggested as a contributing factor to this declining growth rate (Thompson & Duck 2010). This possibility of emigration may be a particularity of North Sea colonies, where overall exponential growth is still witnessed. In colonies elsewhere in Scotland, such as the West coast, where populations are stable but available breeding sites are limited, the role of disease and neonatal mortality on population

dynamics could be very different. To better understand how the overall abundance of grey seals is changing and what role neonatal disease plays in shaping these changes, similar studies should be performed on colonies with contrasting population dynamics for which long term population data are available. If the causes of death and proportion of neonatal mortality were the same in contrasting colonies, neonatal mortality is unlikely to be one of the density dependent factors influencing population growth in grey seals in Scotland.

Several additional interesting conclusions can be drawn from this current data with regards disease within the neonatal Isle of May grey seal population. Choice of pupping ground and substrate was found to significantly influence the presence of bacterial and viral diseases within the colony. Rona rocks, composed of rocky stagnant pools, was the site with the lowest numbers of live pups positive for PhHV1 and *Salmonella* and also showed the highest mass for live pups, suggesting efficient maternal investment. From an anthropomorphic point of view, this site was “dirty” with numerous areas of stagnant water and muddy wallows and finding a lower level of infectious disease on this site was counter intuitive. This site was also relatively further from the sea compared to the others but the abundant pools and unusual topography presumably restricted the movement of pups and mothers resulting in less chance of maternal separation and transmission of infectious diseases from pup to pup or adult seal to non-related pup.

In contrast, pups born on the tidal boulder beach (Pilgrim’s Haven) had the highest prevalence of *Salmonella* and PhHV1, a high proportion of pups succumbing to umbilical infection and the lowest mean pup mass. This finding was surprising given the flushing action of the tides in this location which would be expected to reduce environmental contamination. However, pup density, and therefore interaction, was higher on this colony due to the twice daily high tides forcing them to congregate into a smaller area. This increased the chance of both direct and indirect horizontal transmission of disease and presumably also resulted in higher stress levels. This difference in disease prevalence between the two sites is in agreement with Baker’s studies

25 years previously with less infectious disease found in grey seal pups on Rona Rocks when compared to Pilgrim's Haven (Baker and Baker 1988).

Several other studies have investigated grey seal cow choice of optimal pupping grounds focusing on factors such as proximity to water, density of females and local topography (Pomeroy et al. 2000a; Redman et al. 2001; Twiss et al. 2000; Twiss et al. 2003). The present study suggests that the so-called “topographical cost” of pupping on a site further from the sea may be balanced by a decreased risk of infectious disease through the decreased likelihood of pups interacting with each other or with other cows.

When compared to the earlier study, the present investigation provides a much more detailed evaluation of causes of death in grey seal pups including virology and a much greater specificity of bacteriology. Bacterial infection accounted for 48% of the deaths of grey seal pups on the Isle of May and a large number of bacterial isolates were potentially of terrestrial origin. Several pathogens previously undetected in grey seals were recorded including *Listeria monocytogenes* and a novel *Mycoplasma* spp., and both were associated with significant pathology in grey seal pups. *Listeria monocytogenes* has not been reported in any marine mammal to date and the origin of this zoonotic pathogen, commonly found in terrestrial livestock species, warrants investigation especially given the high prevalence in dead pups in this study (26%) and the similarity between lesions seen in these pups and those seen in systemic listeriosis of neonatal calves, lambs and foals (Maxie & Youssef 2007). In the marine environment this pathogen has previously been described in estuarine water, sediment, shellfish and wild fish species (Colburn et al. 1990; Monfort et al. 1998; Rodas-Suarez et al. 2006), and is suspected to originate from contamination from terrestrial sources (Colburn et al. 1990). Further typing of the *L. monocytogenes* isolates isolated in the present study using pulsed field gel electrophoresis (PFGE), multilocus variable number of tandem repeat analysis (MLVA) and multi-locus sequence type (MLST) schemes may determine the suspected terrestrial origin of this pathogen.

This study also provided a unique opportunity to investigate the prevalence and effect of viral agents, in particular PhHV1 in a wild grey seal breeding colony. The widespread presence of PhHV1 in live and dead grey seal pups (56.2% and 57.8% in dead and live free-ranging grey seal pups, respectively) suggests that most will have been exposed to this virus by the time they leave the colony. This finding is typical of host-adapted alpha herpesviruses in densely populated breeding colonies, such as is seen in breeding catteries with feline herpesvirus 1 (FeHV1) and breeding kennels with canine herpesvirus 1 (CaHV1) (Gaskell et al. 2007; Gaskell & Willoughby 1999). The pathogenicity of PhHV1 is poorly understood but the significant correlation with hepatic necrosis, thymic atrophy and mouth ulcers found in this study points to a similar pathogenesis to that seen in harbour seals (Borst et al. 1986; Goldstein et al. 2005; Himworth et al. 2010). This suggests that, even though grey seal pups have been shown to be less affected by this virus than the sympatric harbour seal (Martina et al. 2002), PhHV1 may be a key component contributing to neonatal mortality both in the wild and in rehabilitation facilities. The key question of what causes the transition between PhHV1 carriage and development of systemic disease in any seal species remains to be elucidated. Host factors such as immunosuppression or age at initial challenge, both factors known to affect the progression and outcome of FeHV1 in cats (Gaskell & Willoughby 1999), are likely to play a part in PhHV1 pathogenesis and should be investigated further.

The study also demonstrated nasal shedding of PhHV1 in yearling grey seals (27.8%) which, given the high prevalence of this virus in pups, is most likely due to stress-induced recrudescence. The initial source of exposure of pups to PhHV1 is unknown but extrapolation from other species and other neonatal alpha herpesviruses would suggest that both perinatal transmission from maternal vaginal secretions and horizontal pup to pup transmission could be involved. Pregnancy associated immuno-suppression has been previously demonstrated in grey seals (King et al. 1994) and the periparturient drop in immunity is a recognised trigger for

transmission of parasites and viruses in numerous domestic animal species (Cattadori et al. 2005; Waller et al. 2004; Xiao et al. 1994). As a result, Goldstein *et al.* (2004) speculated that in harbour seals, cows may be the source of infection through periparturient recrudescence of PhHV1 excreted in nasal and vaginal secretions. To investigate this further in grey seals, nasal swabs and vaginal swabs should be taken from adult females immediately before and after pupping and from adult females out-with the breeding season. If PhHV1 shedding is linked to stress, monitoring nasal viral titres could be used as an indicator of welfare/stress in seals in rehabilitation centres, informing indirectly on the effects of current practices and hygiene measures. Rehabilitation centres would also provide an accessible system in which to study PhHV1 transmission and pathogenesis in grey seal pups as has been performed previously in harbour seals (Goldstein et al. 2004; Goldstein et al. 2005).

This is the first report of PhHV2 in UK grey seals. However, given that it was only found in live yearlings its role in disease remains uncertain and in light of the small sample size so does its true prevalence. To investigate the prevalence in grey seals, a longitudinal study of pups and yearlings, combined with a better diagnostic tool would be required. Gamma herpesviruses tend to be oncogenic and/or immunosuppressive so the effect of infection with PhHV2 on recrudescence of PhHV1 would also be of interest. Furthermore, many gamma herpesviruses tend to have relatively low pathogenicity in their host-adapted species but can lead to severe disease in related host species. This is seen with both ovine herpesvirus 2 and alcelaphine Herpes Virus 1 which naturally and asymptotically infect sheep and wildebeest respectively, yet result in significant disease in the form of malignant catarrhal fever in other ungulates (Widén et al. 2012). This may be a concern with respect to the sympatric relationship between grey and harbour seals.

The lack of *Brucella* spp. in pups is a key finding. This supports two recent papers suggesting that, in both harbour seals and hooded seals, exposure to *B. pinnipedialis* is age-dependent and

most likely to occur post-weaning, during the first year of life, possibly via ingestion of marine fish or invertebrate prey (Lambourn et al. 2013; Nymo et al. 2013). The pathology of this bacterium in seals, if any, has not yet been elucidated, however carriage is widespread with frequent isolation from the lung and mediastinal or pulmonary lymph nodes of juvenile and adult seals (Foster et al. 1996; Lambourn et al. 2013). In contrast, the cetacean associated *Brucella*, *B. ceti*, has been shown to cause a wide range of lesions in several cetacean species (Dagleish et al. 2007; Dagleish et al. 2008; Foster et al. 2007; Gonzalez-Barrientos et al. 2010; Nymo et al. 2011). Given the low odds of juvenile grey seal survival to 1 year of age, it is noteworthy that this period overlaps with the key exposure time of these animals to *Brucella* raising the possibility that this organism may play an as yet unseen role in juvenile mortality. Correlation of thorough post-mortem findings and specialist bacteriology in juvenile grey seals will be critical in exploring this further.

Multidrug resistant *Pseudomonas aeruginosa* were cultured from tissues of three dead pups found both on the Isle of May and in the rehabilitation centre and two isolates of highly multidrug resistance *Salmonella* Typhimurium DT104 were isolated from free-ranging grey seal pups. This raises concerns of antimicrobial resistance transfer into marine mammal populations and the wider marine ecosystem which may recirculate directly into humans or indirectly via livestock.

Studying grey seal pups within a rehabilitation centre provided valuable information regarding pathogen carriage on arrival at the centre, which in turn reflected pathogen diversity over a wide geographical area. Sample size, however, was relatively small and larger numbers would better determine risk factors for exposure to pathogens such as *Salmonella* and *Campylobacter*.

Studying the effects of factors such as human population density, proximity to sewage or freshwater outflow and cumulative precipitation may help investigate the hypothesis that these

infections result from land-sea transfer of terrestrial pathogens. Working with animals in rehabilitation centres has additional limitations due to the likely effects of captivity induced stress and close cohabitation. However, these limitations are balanced by the accessibility of this population and the ability to perform longitudinal studies on these animals. Future studies would employ systematic sampling of pups on arrival and on departure with an emphasis on screening for development of antimicrobial resistance.

Most wildlife disease investigations have inherent limitations and this study was no exception. Firstly, as this work only focused on a single breeding season and a single site, care should be taken in extrapolating the findings to successive seasons and sites. Replication of this study over several seasons or at regular intervals would determine the validity of these results.

Sex, mass and condition at weaning have been shown to determine first year survival (Hall et al. 2002) of grey seals. However, the impact of disease on survival has yet to be determined. This current work represents a cross sectional study of disease and the limiting factor is the inability to follow, chronologically, “apparently healthy” pups and correlate growth, reproductive capacity and survival with the presence of infectious agents. Ideally a longitudinal study of pups, from birth to one year of age, should be performed with repeated sampling for specific pathogens at regular intervals throughout this period. This type of study was not possible in the present case for two reasons. Firstly, a large number of animals would be required for a meaningful study. Assuming a 10% neonatal mortality rate, 500 grey seal pups would have to be studied at birth in order to obtain 50 pups at post-mortem examination. Secondly, longitudinal studies of neonatal pups may result in artificially increased mortality through risk of maternal separation and increased stress due to intervention for sampling.

Given the significance of placental examination in the investigation of reproductive disorders in farm animal species, placental examination was envisaged to be included this study.

Unfortunately, both dead pups and placentas were targets for scavenging seabirds and only three placentas were collected in a suitable condition for inclusion in this study, leaving a large gap to be filled in our knowledge of potential reproductive disease of grey seals. Relevant samples have been taken and stored appropriately should funds become available in the future.

Toxins were a significant but intentional omission from the present study as their inclusion would have been financially prohibitive. Without financial constraints, the levels of fat soluble toxins in these pups, transferred during lactation, would have been compared to the cause of death or immune status of the pups.

Determination of host-pathogen interaction was also beyond the scope of this study but nonetheless warrants discussion. Genetic host-resistance to disease has been considered in previous studies of grey seals. MHC-DQB allele frequencies have been shown to be more similar in grey seals breeding in similar habitats than in grey seals breeding in different habitats, suggesting a degree of environmental pressure on these genes (Cammen et al. 2011).

Furthermore, low allelic diversity at the MHC-DQB gene is purported to be very strongly associated with low chance of pup survival (de Assuncao-Franco et al. 2012). These authors suggest a near deterministic effect of MHC on survival, a powerful and certainly over-simplistic effect given the diverse causes of death of pups in the present study. In particular, processes such as maternal separation/starvation, omphalitis or stillbirth would be unlikely to each exert the same ecological or selective pressures on a population. The low genetic diversity at the DQB gene of this species may suggest that a different suite of MHC alleles is in play with regard grey seal resistance to disease, possibly the DRB genes. These are, as yet, uninvestigated in grey seals but do show extensive diversity in the New Zealand sea lion pointing to a potential role in disease susceptibility (Osborne et al. 2013).

The present study confirms that there is a high pathogen burden within a grey seal breeding colony which, in turn, may select for a more robust immune response and increased disease resistance after leaving the colony if the pups survive the first few weeks. It would be interesting to have comparable data from harbour seal breeding sites as given their assumed susceptibility to a wide range of pathogens in later life there might be expected to be less pathogen related immune priming during the neonatal period.

A limitation of this study is the reliance on available diagnostic tests, as we can only detect what we suspect may be there based on current knowledge of disease in pinnipeds and other marine mammals. Consequently, assessing the true “disease burden” of each animal remains an abstract concept but represents the ultimate goal of the pathologist. Technologies such as deep sequencing and high-density microarrays may help detect elusive and as yet unknown pathogens, but will bring considerable challenges. Current applications include: the faecal virome of California sea lions which has revealed the presence of numerous enteric viruses, many novel in this species (Li et al. 2011); and both a novel cetacean polyomavirus (Anthony et al. 2013) and a novel B19-like parvovirus in the brain of a harbour seal (Bodewes et al. 2013b). A significant challenge of this technology will be correlating findings with supportive pathological and epidemiological information.

The present study will lead to development of better diagnostic tools for use in disease monitoring of wild and stranded grey seal populations. Based on the two real-time PCR (qPCR) assays described in this thesis (phocine distemper virus and seal poxvirus), the recently published qPCR assays for phocid herpesvirus-1 (Roth et al. 2013) and marine caliciviruses (Reid et al. 2007), a multiplex qPCR assay could be developed. This would provide a rapid investigation tool for use in future mass mortality events or in routine disease surveillance.

7.2 Evidence of land-sea transfer of terrestrial pathogens

The zoonotic agents *Salmonella*, *Campylobacter* and *Toxoplasma* were specifically targeted and successfully detected in this study and their putative anthropogenic origin was investigated using a wide range of molecular techniques including cutting-edge comparative genomics to determine their origin. Chapters 4 and 5 show compelling evidence that *Campylobacter* and *Salmonella* isolates found in grey seal pups are shared with humans and other terrestrial mammals and in the case of *Campylobacter* it is possible that humans may even represent the source of exposure of grey seals. The implications of these findings are on several levels. The direct effect of these pathogens on marine mammals is of concern. In Chapters 4 and 5, *Campylobacter* and *Salmonella* were shown to cause significant disease in grey seal pups, while the pathogenicity of *Toxoplasma* in this species remains largely unknown (Gajadhar et al. 2004). Additionally, the potential impact on other marine mammal species should also be considered: several cases of fatal salmonellosis have been reported in species such as grey seals, harbour seals and a killer whale (*Orcinus orca*) (Baker et al. 1995; Colegrove et al. 2010; Foster et al. 1998); and clinical toxoplasmosis has been described in a wide range of marine mammal species including harbour seals (Di Guardo et al. 2010; Dubey et al. 2004; Dubey et al. 2006; Holshuh et al. 1985; Mikaelian et al. 2000; Miller et al. 2001b). Although the decline in British harbour seal populations is likely to be multifactorial (Hall & Frame 2010; Hanson et al. 2013; Thompson et al. 2005), environmental microbial pollution may be a contributing factor to their current demise.

The exact source and transmission routes remain to be proven, but the microbial pollution demonstrated in this study may be an indicator of wastewater and agricultural runoff. It is well established that such processes contribute to nutrient load and availability in freshwater and marine ecosystems. In turn, this can lead to blooms of potentially toxic marine algal species

such as *Alexandrium* spp., *Gymnodinium breve* and *Pseudonitzschia australis*, producing saxitoxins, brevetoxin and domoic acid, respectively. Reports of the detrimental effects of these biotoxins on marine mammal species are increasing worldwide with mass mortalities of manatees and bottlenose dolphins following exposure to brevetoxin (Bossart et al. 1998; Twiner et al. 2012) and well established acute and chronic neurological and systemic effects of domoic acid in California sea lions (Scholin et al. 2000; Silvagni et al. 2005; Zabka et al. 2009).

Extending beyond marine mammals, studying the presence of these terrestrial pathogens allows assessment of the health of the wider marine ecosystem. The concept of ecosystem health, especially in the marine environment, is relatively new (Rapport et al. 1998) and was developed as a response to the accumulating evidence that human-dominated ecosystems have become highly dysfunctional (Haberl et al. 2007). As Reddy *et al.* (2001) point out, “The effects of humans can be found in every ecosystem, whether it is the dampest rain forest, high on the most frigid mountain top, or surrounded by the driest desert”. As an integral part of this ecosystem, human wellbeing is dependent on its sustainability. Not only do we rely on the marine ecosystem to provide over 60% of the biosphere’s economic value, direct subsistence for over 200 million people and a vital role in the global carbon cycle (Wilcox & Aguirre 2004) but through recreational activities and consumption of fish and shellfish products humans are directly exposed to chemical and microbial pollution in this habitat. This illustrates the intricate links between human, wildlife and ecosystem health supported by the “One health, One medicine” initiative (<http://www.onehealthinitiative.com/mission.php>).

Documenting evidence of microbial pollution is only of use if acted upon. Collaborations between public health, coastal water policy and marine animal health research should be established if land-sea transfer of pathogens and toxins is to be reduced. The first step is to identify the source of the pollution, then establish mitigation measures, if possible.

Practical steps to investigate the source of this microbial pollution should involve more extensive screening of other potential sources on the colony such as seawater, sediment, adult seals and other host species, such as rabbits, mice or seabirds. In particular, investigating the presence of these pathogens on the colony at times other than the seal pupping season could help elucidate the possibility of bacterial persistence on the colony.

Time constraints prohibited sampling of wild birds during this study but many questions have arisen as to their role in disease transmission. Although wild birds have been shown to carry mostly host adapted strains of *Campylobacter* (de Haan et al. 2013; Griekspoor et al. 2013; Lu et al. 2011) this may not hold true for the Isle of May population. To investigate whether birds represent a reservoir of these pathogens, wild birds, in particular gulls, on the Isle of May could be sampled during the summer months when the grey seals are not breeding to avoid any effect of scavenging and infection from the seals.

The prevalence and types of terrestrial faecal and protozoal pathogens should be compared on seal colonies close to urban centres and in more remote areas. This could help elucidate risk factors for these pathogens, and ultimately instigate effective control or monitoring measures. Extrapolating from the on-going research in *Toxoplasma* in sea otters (Shapiro et al. 2012) it could be hypothesised that the further the colony is from a centre of urbanisation or high levels of fresh-water run-off, the lower the likelihood of exposure to pathogens such as *Toxoplasma* or *Campylobacter*. A wider range of pathogens should be examined such as *Cryptosporidium* spp., and sampling efforts should target different age groups of seals, sea birds and the environment to obtain a better understanding of the origin and ecology of these pathogens.

As destruction of wetland habitats may facilitate pathogen pollution in coastal waters (Shapiro et al. 2010) responses to evidence of microbial pollution could include the creation or extension

of pre-existing wetlands, within farm settlement systems, limiting construction on critical ecosystems which act as sumps for pollutants and pathogens and more novel approaches such as the development of micro-filtration technologies or sponge-submerged membrane bioreactors (SSMBR) in urban areas (Martinez-Sosa et al. 2011).

The question remains ‘could these pathogens be monitored to study improvement of microbiological quality of UK waters?’ Monitoring programs to assess the effectiveness of measures taken to improve water treatment or outflow could focus on *Salmonella* serovars other than Bovismorbificans, and *Campylobacter* spp.. Evidence of an eradication of *Salmonella* Typhimurium and *S. Haifa* isolates and/or a decrease in the diversity of *Campylobacter* sequence types sharing the same source as humans would indicate an improved environmental status. Similarly witnessing a shift from *Campylobacter* isolates of human origin towards wild bird or environmental isolates may indicate decreased pressure from anthropogenic sources. This type of pro-active screening would not preclude monitoring through marine animal stranding schemes, but in this case, sample selection should bear in mind the poor persistence of *Campylobacter* with delay. *Salmonella* and *Toxoplasma* may be more appropriate markers as they are not as sensitive to processing delay and carcass decomposition.

The final question is whether we can truly consider grey seals as sentinels of coastal marine health. The concept of marine mammals being sentinels of the marine environment is widely disseminated (Aguirre & Tabor 2004; Bossart 2011; Moore 2008) but whether they represent true “sentinels” or rather indicators of marine health is worthy of debate.

Grey seals are charismatic megafauna, living at the interface between the terrestrial and marine environment, they are top predators and long lived. This study has shown that they are exposed to environmental stressors such as bacterial and protozoal agents from the terrestrial ecosystem at a very early stage in their lives. Furthermore, they are relatively accessible, spending a large proportion of time around the near-shore or hauled out on land. . Unfortunately, one proposed

disadvantage of using marine mammals as sentinels/indicators of coastal marine health is their migratory nature and the connection of the World's oceans, making it hard to pin point the exact source of exposure to the toxin or pathogen (Stewart et al. 2008). This is certainly true for adult seals but grey seal pups are a more static population with infection from environmental sources more likely to reflect their local environment than for adult seals. However, do those characteristics make them true sentinels?

The concept of sentinel species was recently reviewed by Halliday *et al.* (2007) who emphasised the lack of consensus as to what defines a “sentinel species” for infectious disease surveillance and described a conceptual framework with which to evaluate the use of a sentinel population depending on the surveillance aim and ecological context. According to these authors, a sentinel population would be assessed according to its response to the pathogen, its relationship with the target population and the existence of transmission routes between the pathogen and the sentinel and target populations (Halliday et al. 2007). Firstly this requires a definition of the target population: in this case, the “coastal marine ecosystem” which would include other marine species whether fish, marine invertebrates, marine mammals or, at a stretch, humans exposed to the marine environment. The spatial overlap of grey seals with this environment is evident but it is important to bear in mind that a large proportion of grey seal pups are born on land where they remain for several weeks before exposure to the marine environment. The second aspect would involve potential transmission routes, which is most likely exposure to sea water, and again the same subset of pups would not fulfil this condition. The final facet would be assessing the “sentinel response to the pathogen”. Although these *Campylobacter* and *Salmonella* do not appear to cause a dramatic host response, the pathogens are readily isolated from rectal swabs of grey seal pups and may be more readily isolated than from samples of seawater.

It could therefore be argued that grey seal pups do not rigorously fit the inclusion criteria to be considered a “sentinel species” for environmental microbial pollution. Nonetheless, they represent an indisputable link between the terrestrial and the marine ecosystems and finding pathogens of anthropogenic origin in these animals is of great concern. Furthermore, these animals offer a window into the likelihood of exposure of other, less accessible, marine mammals to the effects of these pathogens.

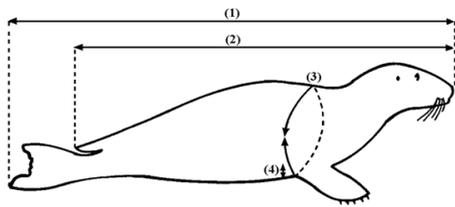
Concluding remarks

This study provides a solid baseline of disease in a grey seal population from which to understand causes of unexpected fluctuations or mass mortality events. It also provides evidence to support land-sea transfer of pathogens of terrestrial origin to grey seals and will hopefully stimulate collaborative trans-disciplinary efforts to achieve a better understanding of the critical links between human activity, ecological change and health.

Appendix 1 - Example of post-mortem record sheet for grey seals used for the study

PM Record sheet – Grey seal project - MRI/SMRU/RZSS

Ref number CD 019 /11 **Sex** Male
Site Gap – crossing to Rona – in sea-water pool **Age** Stillborn
GPS Location Latitude: 56.1896 ; Longitude: -2.5626 **Coat** Whitecoat – Stage 1
Teeth None



Date PM 13/11/11
Pathologist J Baily

Weight (kg)	9.00	Girth (cm)	45
Length 1 (cm)	97	Blubber (mm)	10
Length 2 (cm)	87		

Carcass condition 2b Good
Body condition Adequate

External examination:

Haircoat Normal
Skin NAD
Nails NAD

Body orifices:

Mouth/teeth Not yet erupted
Nose small amount of white frothy fluid
Eye(L) Exophthalmos, marked. Markedly swollen and congested (photo); white lenses – look like cataracts (opaque centre – presumed cold artefact). Severe bilateral periocular haemorrhage
Eye (R) As L
Ear (L) NAD
Ear (L)
Genital mucosa NAD
Anus

Appendix 1 – Post-mortem record sheet

Subcutis	Marked congestion/haemorrhage/oedema –extending over neck/masseters/eyes and larynx extending into musculature surrounding C1 and C2; Haemorrhage over bridge of nose and eyes – small haemorrhages within the temporal muscle		
Blubber	NAD		
Peripheral LN	NAD		
Skeletal muscle	NAD		

Abdominal cavity

General (ascites/position organs...)

Umbilicus	Remnant of umbilical cord present? Yes	10cm long, pale pink
	Internally – normal (no infection)	
Peritoneum	Mildly congested	
Liver	Brown/red	
Gall bladder	NAD	
Spleen	Mildly congested – soft, flaccid	
MLN	NAD	
Pancreas	NAD	
Stomach	NAD	
Duodenum	NAD	
Small intestine	NAD	
Large intestine	Moderate amount of brown pasty meconium	
ICC junction	NAD	

Urinary tract

Adrenal (L)	Small – not weighed	Adrenal (R)	NAD
Kidney (L)	NAD	Kidney (R)	NAD
Ureter (L)	NAD	Ureter (R)	NAD
Bladder/urethra	NAD		

Reproductive organs

Ovary/oviduct	NA	Testicle/vas	NAD
Uterus/cervix	NA	Penis/os penis	NAD
Vagina/vestibule	NA	Prepuce	NAD

Thoracic cavity

General (effusion/adhesions...)

Diaphragm	NAD		
	Moderately congested ventrally – with interlobular haemorrhages. Marked, diffuse oedema within interlobular septae and between lobes; small areas of emphysema (on dorsal aspect), bubbly on cut section and FLOATS		
Lung (L)		Lung (R)	floats

Ribs	NAD	Pleura	NAD
Thymus	Medium sized – not able to weigh	Mediastinal LN	NAD

Neck

Thyroid/PTH (L)	NAD	Thyroid /PTH (R)	NAD
Oesophagus	NAD	Tongue	NAD
Larynx	NAD	Tonsils	NAD
Trachea	Moderate amount of stable white froth		
Mainstem bronchi	Stable white froth		Bronchioles NAD

Heart

Pericardium	NAD	Blood	
Myocardium	NAD	Aorta	NAD
Valves	NAD	Other vessels	NAD

CNS

Brain	Mild congestion – mildly oedematous on ventral aspect		
Pituitary gland	NAD		
Peripheral nerves	NAD		
Trigeminal ganglia	NAD		

Skull

Nasal cavities/choana	Nasal mites	No
Nasal sinuses	NAD	

Gross diagnosis - Cause of death: Stillborn – dystocia

Severe haemorrhage and oedema of head and neck;
 exophthalmos;
 pulmonary oedema (asphyxiation)

Other notes

Ref number CD019/11**SAMPLES**

External examination	Virology	Bacteriology	10% NBF	Other	Weight
A Nasal swab	Swab	Swab			
B Rectal swab		Swab x2			

Abdominal cavity

1 Liver	P		1		g
2 Spleen	P		1		g
3 MLN	P		1		
4 Pancreas			1		
5 Stomach			1		
6 Small intestine			1		
7 Ileo-caeco-colic junction	P		1		
8 Large intestine			2		
9 Kidney	P		1		g
10 Adrenal			1		g
11 Urinary bladder			2 (nick)		
12 Vagina/ Penis - mucosa			2		
13 Testis/ Ovary			2		

Thoracic cavity

21 Left cranial lung lobe	P		1	Toxo pot	
22 Right caudal lung lobe			2		
23 Bronchial lymph node	P		Bijou 2		
24 Trachea			1		
25 Diaphragm			1		
26 Right ventricle	P		2	Toxo pot	g
27 Left ventricle			1		
28 Thymus			2		g
29 Oesophagus			2		

Head/neck

31 Thyroid			1		
32 Tonsil	P		2		
33 Tongue			1	Toxo pot	
34 Buccal mucosa - cheek			1		
35 Skin – cheek			1	2cm ² Frozen	
36 Salivary gland			2		
37 Retropharyngeal lymph node			2		

Nervous system

41 Brain	P		2	Toxo pot - hind	g
42 Pituitary gland			Cassette W		
43 Trigeminal ganglion			Cassette G		
44 Cervicothoracic ganglion			Cassette O		
45 Spinal ganglion			Cassette Red		

Other tissues

51 Bone marrow - rib			2		
52 Eye - Right			1		

Additional samples if seen

61 Mouth ulcers			Orange pots		
62 Skin lesions			Orange pots		

Body fluids

71 Blood plasma	
72 Buffy coat	
73 Urine	

**Sampling
Key**

	sampled
	not to be sampled
X	not taken

Appendix 2 – Detailed results of genome sequencing of 90 *Campylobacter* isolates from grey seals

Table provides isolate number, isolate reference, attribution of MLST allele numbers, sequence types (ST) and clonal complex (CC) (based on the *C. jejuni* and *C. coli* MLST scheme). The number of contigs and N50 values obtained using both de novo genome assembly and reference based genome assembly are provided, along with the reference sequence used for reference based assembly. The presence (1) or absence (0) of the hippuricase gene (HipO) is specified along with the allele number of the HipO gene (if present).

Isolate Nb	Isolate reference	MLST gene alleles							ST	CC	De novo assembly		Reference based assembly			HipO gene	
		aspA	glnA	gltA	glyA	pgm	tkr	uncA			Nb Contigs de novo	N50 de novo	Assembly reference sequence	Nb contigs	N50	HipO gene	HipO allele
1	A003	4	7	10	4	1	7	1	45	ST-45	96	39929	M1	70	70324	1	2
2	A007	104	7	10	4	1	7	1	1326	ST-45	88	53657	M1	69	63206	1	2
4	A018	33	39	30	82	104	56	17	827	ST-828	56	101682	11168	96	78195	0	NA
5	CD008 1/3	4	7	10	4	42	51	1	583	ST-45	73	40919	M1	59	56209	1	5
9	CD009 2/3	4	7	10	4	42	51	1	583	ST-45	96	36098	M1	55	60785	1	5
11	CD012 3/3	4	7	10	4	1	7	1	45	ST-45	125	31881	M1	90	58220	1	2
14	CD013 1/3	4	7	10	4	42	51	1	583	ST-45	43	121702	M1	60	56209	1	5
18	CD016 3/3	4	7	10	4	42	51	1	583	ST-45	96	33433	M1	53	59762	1	5
21	CD017 2/3	4	7	10	4	42	51	1	583	ST-45	32	121702	M1	66	55482	1	5
26	CD018 1/3	2	1	1	3	2	1	5	21	ST-21	153	29277	11168	43	130265	1	10
28	CD022 1/2	2	1	21	334	2	1	5	3853	ST-21	86	86230	11168	40	155633	1	9
29	CD024 2/2	4	7	10	4	1	7	1	45	ST-45	94	57656	M1	59	117964	1	2
30	CD024 1/2	1	3	6	4	3	3	3	22	ST-22	70	66451	11168	83	70801	1	27
31	CD025 2/3	4	7	10	4	42	51	1	583	ST-45	32	144235	M1	59	55482	1	5
32	CD025 1/3	4	7	10	4	42	51	1	583	ST-45	72	60131	M1	66	53568	1	5

Appendix 2 – Sequence types and MLST alleles of 90 *Campylobacter* isolates

Isolate Nb	Isolate reference	MLST gene alleles							ST	CC	De novo assembly		Reference based assembly			HipO gene	
		aspA	glnA	gltA	glyA	pgm	tkr	uncA			Nb Contigs de novo	N50 de novo	Assembly reference sequence	Nb contigs	N50	HipO gene	HipO allele
34	CD027 2/3	1	3	6	4	3	3	3	22	ST-22	231	14370	11168	117	46565	1	27
37	CD028 2/3	1	3	6	4	3	3	3	22	ST-22	215	17683	11168	115	42694	1	27
39	CD029 2/3	1	3	6	4	3	3	3	22	ST-22	340	9668	11168	144	28695	1	27
42	CD031 2/3	2	1	21	334	2	1	5	3853	ST-21	84	103275	11168	60	79993	1	9
43	CD031 1/3	2	1	1	3	2	1	5	21	ST-21	233	15737	11168	60	105874	1	10
45	CD032 1/3	2	1	1	3	2	1	5	21	ST-21	68	71580	11168	42	189475	1	10
46	CD032 2/3	2	1	1	3	2	1	5	21	ST-21	140	32234	11168	41	135317	1	10
48	CD033 2/3	2	1	12	3	2	1	5	50	ST-21	85	48045	11168	49	96938	1	10
52	CD034 2/2	2	1	1	3	2	1	5	21	ST-21	176	22241	11168	45	178219	1	10
55	CD036 3/3	33	39	30	82	104	56	17	827	ST-828	183	19225	11168	92	78112	0	RC
56	CD037 1/3	2	1	12	3	2	1	5	50	ST-21	340	10019	11168	103	45551	1	10
60	CD038 3/3	33	39	30	82	104	56	17	827	ST-828	62	132412	11168	105	78195	0	NA
61	CD039 2/3	33	39	30	82	104	56	17	827	ST-828	51	146954	11168	103	78195	0	NA
64	CD046 1/2	33	39	30	82	104	56	17	827	ST-828	44	162171	11168	105	78195	0	NA
66	CD048 2/3	33	39	30	82	104	56	17	827	ST-828	50	163522	11168	104	78195	0	NA
69	CD050 3/3	33	39	30	82	104	56	17	827	ST-828	59	163559	11168	107	78195	0	NA
72	CL005 1/2	22	335	4	64	74	25	23	4001	ST-1034	64	73654	11168	71	57292	1	67
73	CL011 1/1	2	165	73	147	220	190	104	1457	ND	64	104234	11168	83	43228	0	RC
76	CL012 3/3	4	7	10	4	42	51	1	583	ST-45	106	32467	M1	70	56209	1	5
77	CL013 1/1	4	7	10	4	42	51	1	583	ST-45	58	68239	M1	58	56209	1	5
79	CL014 2/2	4	7	10	4	42	51	1	583	ST-45	107	31094	M1	65	64143	1	5
81	CL015 2/2	4	7	10	4	42	51	1	583	ST-45	49	87669	M1	48	80610	1	10
82	CL016 1/2	2	1	12	3	2	1	5	50	ST-21	60	112467	11168	72	60116	1	10

Appendix 2 – Sequence types and MLST alleles of 90 *Campylobacter* isolates

Isolate Nb	Isolate reference	MLST gene alleles							ST	CC	De novo assembly		Reference based assembly			HipO gene	
		aspA	glnA	gltA	glyA	pgm	tkt	uncA			Nb Contigs de novo	N50 de novo	Assembly reference sequence	Nb contigs	N50	HipO gene	HipO allele
85	CL017 2/2	10	8	34	6	39	88	3	1256	ND	105	65177	11168	126	47551	0	NA
87	CL018 2/2	2	1	12	3	2	1	5	50	ST-21	76	54355	11168	63	130698	1	10
88	CL019 1/2	2	1	4	28	58	25	58	696	ST-1332	84	77224	11168	105	76485	1	38
90	CL020 1/2	1	3	6	4	3	3	3	22	ST-22	83	45996	11168	81	75693	1	27
91	CL020 2/2	1	3	6	4	3	3	3	22	ST-22	243	13624	11168	105	40647	1	27
92	CL021 1/2	4	7	10	4	1	7	1	45	ST-45	71	64998	M1	74	82893	1	2
95	CL022 2/2	4	7	10	4	1	7	1	45	ST-45	79	82108	M1	75	70269	1	2
97	CL023 2/2	4	7	10	4	1	7	1	45	ST-45	64	121093	M1	68	66724	1	2
98	CL026 1/2	4	7	10	4	1	7	1	45	ST-45	55	101805	M1	73	60055	1	2
99	CL026 2/2	4	7	10	4	1	7	1	45	ST-45	103	38784	M1	72	63576	1	2
100	CL027 1/2	4	7	10	4	1	7	1	45	ST-45	73	84387	M1	74	60055	1	2
101	CL027 2/2	4	7	10	4	1	7	1	45	ST-45	62	94928	M1	71	63671	1	2
102	CL030 1/3	4	7	10	4	1	7	1	45	ST-45	82	66324	M1	70	84599	1	2
107	CL039 1/3	2	1	21	334	2	1	5	3853	ST-21	78	90251	11168	51	109900	1	9
109	CL040 2/2	NA	NA	NA	NA	NA	NA	NA		<i>C. lari</i>	87	201291	RM2100	54	201261	0	NA
110	CL042 3/3	33	39	30	82	104	47	36	962	ST-828	73	82506	11168	101	79580	0	NA
113	CL043 2/3	2	1	12	3	2	1	5	50	ST-21	80	50581	11168	51	1673797	1	10
116	CL044 2/3	1	3	6	4	3	3	3	22	ST-22	67	84191	11168	102	70788	1	27
117	CL044 1/3	1	3	6	4	3	3	3	22	ST-22	66	86330	11168	70	93451	1	27
118	CL044 3/3	1	3	6	4	3	3	3	22	ST-22	52	145434	11168	76	55951	1	27
120	CL047 2/3	33	39	30	82	104	56	17	827	ST-828	50	265644	11168	104	78795	0	NA
122	CL056 2/2	4	7	10	4	42	51	1	583	ST-45	33	150853	M1	49	69124	1	5
123	CL056 1/2	4	7	10	4	42	51	1	583	ST-45	38	144414	M1	45	1601038	1	5

Appendix 2 – Sequence types and MLST alleles of 90 *Campylobacter* isolates

Isolate Nb	Isolate reference	MLST gene alleles							ST	CC	De novo assembly		Reference based assembly			HipO gene	
		aspA	glnA	gltA	glyA	pgm	tkt	uncA			Nb Contigs de novo	N50 de novo	Assembly reference sequence	Nb contigs	N50	HipO gene	HipO allele
124	CL058 3/3	4	7	10	4	1	7	1	45	ST-45	66	75139	M1	73	80071	1	2
126	CL058 1/1	4	7	10	4	1	7	1	45	ST-45	63	85647	M1	83	47734	1	2
129	CL061 1/3	33	39	30	82	104	56	17	827	ST-828	51	163520	11168	101	78195	0	NA
130	CL061 2/3	33	39	30	82	104	56	17	827	ST-828	50	163559	11168	79	78185	0	NA
132	CL062 3/3	2	1	21	334	2	1	5	3853	ST-21	76	90251	11168	40	138347	1	9
133	CL062 1/3	2	1	21	334	2	1	5	3853	ST-21	78	103199	11168	54	109576	1	9
135	CL063 3/3	4	7	10	4	1	7	1	45	ST-45	48	123138	M1	69	56553	1	2
138	CL064 1/3	2	1	21	334	2	1	5	3853	ST-21	85	106473	11168	30	182902	1	9
141	CL065 1/1	33	39	30	82	104	56	17	827	ST-828	45	170018	11168	102	78175	0	NA
142	CL067 3/3	2	1	21	334	2	1	5	3853	ST-21	81	115491	11168	56	88926	1	9
146	CL069 2/2	2	1	21	334	2	1	5	3853	ST-21	83	105241	11168	49	69839	1	9
147	CL070 1/1	2	1	21	334	2	1	5	3853	ST-21	82	115491	11168	55	78324	1	9
149	CL071 2/2	1	3	6	4	3	3	3	22	ST-22	50	151228	11168	59	86910	1	27
151	CL072 2/3	1	3	6	4	3	3	3	22	ST-22	99	39565	11168	75	105835	1	27
153	CL074 1/3	33	39	30	82	112	56	17	1578	ST-828	71	162389	11168	113	79866	0	NA
156	CL075 2/3	4	7	10	4	1	7	1	45	ST-45	46	132576	M1	56	1618132	1	2
161	CL076 3/3	4	7	10	4	1	7	1	45	ST-45	86	60004	M1	71	77449	1	2
162	CL078 1/3	1	3	6	4	3	3	3	22	ST-22	50	151228	11168	85	67718	1	27
165	CL079 1/3	1	3	6	4	3	3	3	22	ST-22	56	127383	11168	79	75726	1	27
170	CL080 3/3	4	7	10	4	42	7	1	137	ST_45	39	122311	M1	39	117337	1	2
171	CL085 1/3	33	39	30	82	104	56	17	827	ST-828	54	135003	11168	103	75018	0	NA
174	CL086 1/3	2	1	12	3	2	1	5	50	ST-21	57	106434	11168	57	103359	1	10
179	CL087 3/3	8	7	4	4	125	7	1	1003	ST-45	158	28510	M1	92	70475	0	NA

Appendix 2 – Sequence types and MLST alleles of 90 *Campylobacter* isolates

Isolate Nb	Isolate reference	MLST gene alleles							ST	CC	De novo assembly		Reference based assembly			HipO gene	
		aspA	glnA	gltA	glyA	pgm	tkr	uncA			Nb Contigs de novo	N50 de novo	Assembly reference sequence	Nb contigs	N50	HipO gene	HipO allele
181	CL088 2/3	2	1	12	3	2	1	5	50	ST-21	69	91564	11168	71	103359	1	10
183	CL090 1/3	2	1	12	3	2	1	5	50	ST-21	88	63724	11168	62	104439	1	10
186	R006	104	7	10	4	1	7	1	1326	ST-45	49	97515	M1	52	82901	1	2
187	RR1 1/3	1	3	6	4	3	3	3	22	ST-22	101	40490	11168	99	69172	1	27
188	RR1 2/3	NA	NA	NA	NA	NA	NA	NA		<i>C. lari</i>	59	133897	RM2100	49	126425	0	NA
190	RR2 1/1	4	7	10	4	1	7	1	45	ST-45	74	66225	M1	52	104814	1	2

Appendix 3 – Novel alleles of *Campylobacter lari*

Three novel alleles of *Campylobacter lari* were determined and submitted to the pubMLST Non *jejuni/coli* *Campylobacter* database: **C^{la}tktnovel1**, **C^{la}tktnovel2** and **C^{la}pgmnovel1**.

Appendix 3 – Novel alleles of *C. lari*

For isolate 109, **C^{la}tktnovell1** is proposed:

> **C^{la}tktnovell1**

```
TTACATTTGAGTGGTTATGATATAAGTTTAGAAGATTTAAAAAACTTCCGTCAATTA
CACTCTAAAACCCCTGGACATCCTGAAATTTTTACCTCAGGTGTTGAAATAGCTACA
GGGCCTTTAGGGCAAGGTGTGGCAAATGCAGTTGGTTTTGCAATGGCAGCTAAAAA
AGCAAGCTTGCTTTTTGGGTGAGGATATTATCAATCATAAAGTGTATTGTTTGTGTGG
TGATGGAGATTTGCAAGAGGGAATTCATATGAAGCTTGCTCTTTAGCTGGGCTTC
ATAAGCTTGATAATTTAATCATCATTATGATAGTAATAATATTTCAATCGAAGGTG
ATGTGGCTATTGCTTTTAATGAAAATGTAAAAGAGCGTTTTAGGGCTCAAATTTTG
AAGTACTTGAGATAGATGGGCATGATTTTGAGCAAATTGATTTAGCGCTTAAAACA
GCTAAG
```

Single base-pair difference compared to C^{la}tk allele 4: Thymidine to Cytosine substitution at nucleotide 275

The screenshot shows a sequence alignment interface. At the top, there is a 'Translate query' button and the text 'Closest match: P*tk: 4'. Below this, a sequence alignment is displayed. The query sequence is shown in green and the reference sequence in black. The alignment is broken into lines of 10 nucleotides each, with positions 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450 indicated above the sequences. A single difference is highlighted at position 275, where the query has 'C' and the reference has 'T'. Below the alignment, a 'Differences' section states '1 difference found.' and shows the substitution '275T → 275C'. At the bottom, it notes 'The locus start point is at position 1 of your query sequence.'

For isolate 188, **C^{la}tktnovel2** is proposed:

> **C^{la}tktnovel2**

```
TTACATTTGAGTGGTTATGATGTAAGTTTAGATGATTTAAAAAATTTCCGTCAATTA
CACTCTAAAACCCCAGGTCATCCTGAAATTTTTACCTCAGGTGTTGAAATCGCCACA
GGGCCTTTAGGGCAAGGTGTGGCAAATGCAGTTGGTTTTGCTATGGCAGCTAAAAA
AGCAAGCTTGCTTTTGGGTGAGGATATTATCAATCATAAAGTGTATTGTTTGTGTGG
TGATGGGGATTTGCAAGAGGGAATTTTCATATGAGGCTTGCTCTTTAGCTGGGCTTCA
TAAGCTTGATAATTTAATTATCATTTATGATAGTAATAATATTTCAATCGAAGGTGA
TGTAGCTATTGCTTTTAATGAAAATGTAAAAGAGCGTTTTAGGGCTCAAAATTTTGA
AGTACTTGAGATAGATGGGCATGATTTTGAACAAATTGATTTAGCACTTAAAACAG
CTAAG
```

Single base-pair difference compared to C^{la}tk allele 6: Adenosine to Guanine substitution at nucleotide 126

Translate query

Closest match: **C^{la}tk: 6**

	10	20	30	40	50	60	70	80	90	100
Query	TTACATTTGAGTGGTTATGATGTAAGTTTAGATGATTTAAAAAATTTCCGTCAATTA									
Ref									
	110	120	130	140	150	160	170	180	190	200
Query	TGAAATCGCCACAGGCGCTTTAGGGCAAGGTGTGGCAAATGCAGTTGGTTTTGCTATGGCAGCTAAAAAGCAAGCTTGCTTTTGGGTGAGGATATTAT									
RefA.....									
	210	220	230	240	250	260	270	280	290	300
Query	CAATCATAAAGTGTATTGTTTGTGTGGTGATGGGGATTTGCAAGAGGGAATTTTCATATGAGGCTTGCTCTTTAGCTGGGCTTCATAAGCTTGATAATTTA									
Ref									
	310	320	330	340	350	360	370	380	390	400
Query	ATTATCATTTATGATAGTAATAATATTTCAATCGAAGGTGATGTAGCTATTGCTTTTAATGAAAATGTAAAAGAGCGTTTTAGGGCTCAAAATTTTGAAG									
Ref									
	410	420	430	440	450					
Query	TACTTGAGATAGATGGGCATGATTTTGAACAAATTGATTTAGCACTTAAAACAGCTAAG									
Ref									

Differences

1 difference found.

¹²⁶A → ¹²⁶G

The locus start point is at position 1 of your query sequence.

Appendix 3 – Novel alleles of *C. lari*

For isolate 188, C^{la}**pgmnovel1** is proposed:

>C^{la}**pgmnovel1**

```
GGTTTAAATATCAATGAAAATTGCGGGGCTTTACATCCTTTAAATTTAGCTTTGGAA
GTAAAGAAATTTAGAGCAGATGTGGGCTTTGCCTTTGATGGAGATGCGGATCGTTT
GGTTGTTGTGGATGAAAAAGGCGAAGTAGCTCATGGAGATAGTCTTTTAGGAGTTT
TGGCTTTATTTTTGAAAAACAAGGCAAATTAATCAAGCGTAGTAAGCACTATA
ATGAGTAATGGTGCTTTAAAAGAGTTTTTAACCAAGCACAAAATTCCACATGAAAC
TTGCAATGTAGGTGATAAATATGTGCTTGAAAAGCTCAAAGAATGTGGCGGAAATT
TTGGCGGGGAGCAAAGTGGGCATATTATTTTTAGTGACTATGCAAAAAGTGGAGAT
GGTTTGGTAGCTGCTTTGCAATTTAGCGCTTTAATGCTTAGTGAAGCAAAAAGCGC
AAGCGAAATTTAAATCAAGTTAAGCCTTACCCGCAACTTTTACATAAT
```

Single base-pair difference: Thymidine to Cytosine substitution at nucleotide 420

Translate query

Closest match: C^{la}pgm: 1

10	20	30	40	50	60	70	80	90	100
Query	GGTTTAAATATCAATGAAAATTGCGGGGCTTTACATCCTTTAAATTTAGCTTTGGAA								
Ref								
110	120	130	140	150	160	170	180	190	200
Query	ATGCGGATCGTTTGGTTGTTGGATGAAAAAGGCGAAGTAGCTCATGGAGATAGTCTTTTAGGAGTTTGGCTTTATTTTTGAAAAACAAGGCAAATT								
Ref								
210	220	230	240	250	260	270	280	290	300
Query	AAAATCAAGCGTAGTAAGCACTATAATGAGTAATGGTGCTTTAAAAGAGTTTTTAACCAAGCACAAAATTCCACATGAAACTTGCAATGTAGGTGATAAA								
Ref								
310	320	330	340	350	360	370	380	390	400
Query	TATGTGCTTGAAAAGCTCAAAGAAATGTGGCGGAAATTTTGGCGGGGAGCAAAGTGGGCATATTATTTTTAGTGACTATGCAAAAAGTGGAGATGGTTTGG								
Ref								
410	420	430	440	450	460	470	480	490	
Query	TAGCTGCTTTGCAATTTAGCGCTTTAATGCTTAGTGAAGCAAAAAGCGCAAGCGAAATTTAATCAAGTTAAGCCTTACCCGCAACTTTTACATAAT								
RefT.....								

Differences

1 difference found. []

⁴²⁰T → ⁴²⁰C

The locus start point is at position 1 of your query sequence. []

Presentations and publications arising from this thesis

Presentations

01/09/11 – **Pathology of Marine Mammals – an Introduction** – 1 hour seminar - British Society of veterinary Pathologists Seminar Series, Roslin Institute, Edinburgh

Sept 2011 - **Pathology Image Group International** – Contributed a training tool for pathology residents focusing on gross pathology of marine mammals

11/02/12 Presented two practical sessions on "**Post mortem examination of Wildlife**" at the Edinburgh Veterinary Zoological Society annual conference

14/06/12 **Prevalence of pathogens in Scottish grey seals** – Farne islands advisory committee, Seahouses, Northumberland

26/07/12 **Prevalence of *Salmonella enterica* and *Campylobacter* spp. in wild caught and stranded neonatal and juvenile grey seals (*Halichoerus grypus*) in Scotland** – Poster presented at Wildlife Disease Association conference in Lyon, France

02/08/12 **Prevalence of pathogens in Scottish grey seals** – Fishcross Wildlife Centre, Alloa

11/09/12 **Prevalence of pathogens in Scottish grey seals** – Scottish Agricultural College seminar series

06/11/12 **Grey seal pathogens ... or “Why it’s probably a good idea to keep your mouth closed while swimming in the sea”** – Moredun Lunchtime Research Seminar - 1 hour talk

31/10/12 **Prevalence of *Salmonella enterica* and *Campylobacter* spp. in wild caught and stranded neonatal and juvenile grey seals (*Halichoerus grypus*) in Scotland** – Poster presented at “Celebrating 20 years of the Scottish marine mammal animal stranding scheme” - National Museum of Scotland, Edinburgh

22/03/13 **Healthy seals, healthy seas? A study of disease in Scottish grey seals** – 15 minute oral presentation at St Andrews Post graduate conference

08/04/13 **Prevalence of *Salmonella enterica* and *Campylobacter* spp. in wild caught and stranded neonatal and juvenile grey seals (*Halichoerus grypus*) in Scotland** Poster presented at 27th Conference of the European Cetacean Society Setubal, Portugal

25/04/13 **Prevalence of *Salmonella enterica* and *Campylobacter* spp. in wild caught and stranded neonatal and juvenile grey seals (*Halichoerus grypus*) in Scotland** 15 minute oral presentation at International Association of Aquatic Animal Medicine 44th Annual Conference Proceedings, April 2013, Sausalito, California

02/09/13 **Causes of grey seal pup mortality and morbidity.** 15 minute oral presentation at the Special Committee on Seals Meeting, 2013, St Andrews

15/11/13 **Healthy seals, healthy seas? A study of disease in Scottish grey seals.** One hour lunchtime seminar at Royal Zoological Society of Scotland, Edinburgh Zoo

Scientific publications arising from this thesis

Prevalence and Risk Factors for Infection with Pathogenic Faecal Bacteria in Scottish grey seals (*Halichoerus grypus*)

Johanna Baily, Geoff Foster, Derek Brown, John Coia, Nick Davison, Simon Moss, Eleanor Watson, Kim Willoughby, Ailsa Hall and Mark Dagleish

Briefing paper to be included in the Special Committee on Seals (SCOS) report 2014

SCOS provides formal advice to the government on matters related to the management of seal populations. Advice is given annually based on the latest scientific information provided to SCOS by the Sea Mammal Research Unit.

<http://www.smru.st-andrews.ac.uk/pageset.aspx?psr=411>

Evidence of land-sea transfer of a zoonotic human pathogen, *Campylobacter* spp., to a wildlife marine sentinel species, the grey seal (*Halichoerus grypus*) – manuscript in preparation

Johanna Baily, Guillaume Méric, Sion Bayliss, Ben Pascoe, Geoff Foster, Simon Moss, Eleanor Watson, Jane Mikhail, Robert Goldstone, Romain Pizzi, David G.E. Smith, Kim Willoughby, Ailsa Hall, Samuel K. Sheppard, Mark Dagleish

Scientific publications not arising directly from this thesis

Infection due to *Mycobacterium avium* subsp. *avium* in a Free-ranging Common Seal (*Phoca vitulina*) in Scotland - Journal of Wildlife Diseases, 49 (3), 2013, pp. 732–734

Geoffrey Foster, Karen Stevenson, Robert J. Reid, Jason P. Barley, **Johanna L. Baily**, Robert N. Harris, and Mark P. Dagleish

Primitive Neuroectodermal Tumour in a Striped Dolphin (*Stenella coeruleoalba*) with Features of Ependymoma and Neural Tube Differentiation (Medulloepithelioma) - Journal of Comparative Pathology 149, 2013, pp. 514-519

J. L. Baily, L. R. Morrison, I. A. Patterson, C. Underwood and M. P. Dagleish

Investigation into the long-finned pilot whale mass stranding event, Pittenweem, Fife, 2nd September 2012. Report to Marine Scotland November 2013

Andrew Brownlow, **Johanna Baily**, Mark Dagleish, Nick Davison, Rob Deaville, Geoff Foster, Silje-Kirstin Jensen, Paul Jepson, Eva Krupp, Robin Law, Barry McGovern, Maria Morell, Rod Penrose, Matt Perkins, Fiona Read

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