An investigation of *Leucocytozoon* in the endangered yellow-eyed penguin (*Megadyptes antipodes*)

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Abstract

Yellow-eyed penguins have suffered major population declines and periodic mass mortality without an established cause. On Stewart Island a high incidence of regional chick mortality was associated with infection by a novel *Leucocytozoon* sp. The prevalence, structure and molecular characteristics of this *leucocytozoon* sp. were examined in the 2006-07 breeding season. In 2006-07, 100% of chicks (n=32) on the Anglem coast of Stewart Island died prior to fledging. Neonates showed poor growth and died acutely at approximately 10 days old. Clinical signs in older chicks up to 108 days included anaemia, loss of body condition, subcutaneous ecchymotic haemorrhages and sudden death. Infected adults on Stewart Island showed no clinical signs and were in good body condition, suggesting adequate food availability and a potential reservoir source of ongoing infections. A polymerase chain reaction (PCR) survey of blood samples from the South Island, Stewart and Codfish Island found *Leucocytozoon* infection exclusively on Stewart Island. The prevalence of *Leucocytozoon* infection in yellow-eyed penguin populations from each island ranged from 0-2.8% (South Island), to 0-21.25% (Codfish Island) and 51.6-97.9% (Stewart Island). The high prevalence on Stewart Island represented the infection of 100% of chicks and 83% of adult yellow-eyed penguins when tested by PCR. Sequencing of *Leucocytozoon* sp. DNA found similarities between infections in yellow-eyed penguin adults and chicks, but differences to *Leucocytozoon* sp. DNA obtained from Fiordland crested penguins. These findings support the suggestion of cross infection between adults and chicks, and indicate that endemic infection in yellow-eyed penguins is unrelated to that in Fiordland crested penguins. Examination by histology and electron microscopy showed tissue megaloschizonts and circulating round

gametocytes. Megaloschizonts up to 440μm diameter showed an affinity for hepatic and splenic tissue and were observed releasing occasional intact cytomeres. Round gametocytes were observed within leucocytes in visceral sections, but not peripheral blood smears. The morphology of *Leucocytozoon* sp. in yellow-eyed penguins showed similarities to the pathogenic species *L. simondi* and *L. sakharoffi* but not *L. tawaki*. A successful treatment protocol for leucocytozoonosis has not been established, although treatment in a Fiordland crested penguin was able to suppress parasitaemia. The role of *Leucocytozoon* in yellow-eyed penguins as a cause of morbidity and mortality remains unclear. Further investigation into direct pathogenicity, and the interaction of concurrent disease and environmental influences is required. The findings of this thesis provide potential management recommendations and highlight areas requiring further investigation.

Declaration

The studies presented in this thesis were completed by the author whilst a postgraduate student in the Institute of Veterinary, Animal and Biomedical Sciences, Massey University. I officially state that this is my own work and the views presented are mine, and that the contents have not been submitted for any other degree. I certify that to the best of my knowledge any help received in preparing this thesis and all sources used have been acknowledged in the thesis

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The content and structure of this thesis

This thesis documents the ongoing investigation of a new species of *Leucocytozoon* identified in the yellow-eyed penguin on Stewart Island in 2005. Its prevalence, structure and implications are explored.

The structure of this thesis consists of 3 parts, a summary of current knowledge regarding *Leucocytozoon* and yellow-eyed penguins; a series of 3 scientific papers, as presented for publication, detailing the investigation of *Leucocytozoon* in yellow-eyed penguins and its neighbour, the Fiordland crested penguin; and a discussion of the findings of this research and its impact on the yellow-eyed penguin.

Chapter 1

An investigation of *Leucocytozoon* in the endangered yellow-eyed penguin (*Megadyptes antipodes*): Literature Review

Yellow-eyed Penguins/Hoiho

Yellow-eyed penguins (Megadyptes antipodes) or hoiho are the world's rarest species of penguin. Their habitat is limited to the southeastern coast of New Zealand's South Island, from Banks Peninsula, Oamaru, Otago Peninsula, and the Catlins to Stewart Island and its smaller satellites (Turbott 1990; McKinley 2001). Further offshore colonies are located on Auckland (50°35'S, 169°00'E) and Campbell lslands (52°32'S, 169°10'E) (Turbott 1990).

Yellow-eyed penguins are large, flightless birds weighing 4.3-6.9kg with a distinctive yellow stripe in the feathers across the eyes. They represent one of only 4 penguins endemic to New Zealand, among 13 of the world's 16 penguin species that can be found within New Zealand territories (Heather and Robertson 1996). Yellow-eyed penguins are endangered as a result of their restricted habitat range and seasonal population declines (Birdlife International 2007). These features combined with their relative public accessibility have made them a flagship species for conservation in New Zealand.

Yellow-eyed penguin biology

Yellow-eyed penguins are a long-lived species, with adults surviving up to 20 years and males usually outliving females. The vast majority of losses take place in the first year of life, with 70-85% of juveniles failing to survive the first year (Anon. 1991). Fledglings disperse in February and March, with an estimated half of these dying during their first five weeks at sea (Duignan 2001). During stable years, adult annual losses are usually no greater than 13-14% (Anon. 1991).

Yellow-eyed penguin reproductive biology has been well documented in accordance with its endangered status. Breeding begins at 2-3 years of age for females and 2-5 years of age for males, with a 2:1 male to female population bias developing in 10-12 year olds (Richdale 1957). Mating takes place in late August after the re-establishment of each pair's annual nesting site in July. Clutches of 1-2 eggs are laid in September which will hatch approximately 39-51 days later in early November (Darby and Seddon 1990). Chicks are fledged at 106 days old in February the following year. Yellow-eyed penguins have a low divorce rate (13%) and share parental duties including nesting, brooding and feeding of chicks. Annual chick production is highly variable between pairs and seasons, but yellow-eyed penguins produce up to 1.4 chicks per pair each year (Moore 1992a). This reproductive rate is high compared to other penguin species that lay two egg clutches and allows populations to respond to downward population pressure through recruitment, while adult longevity sustains populations through unstable years (Darby and Seddon 1990; Moore 1992a).

Habitat

Yellow-eyed penguins are dependent on the integrity of both marine and coastal habitats for survival. The marine environment represents a source of food and a means of transport and dispersal (McKinley 2001). Foraging studies at Otago Peninsula found that radio-tagged birds travelled 3-25km offshore or 5-35km from breeding areas, usually in waters 30-70m deep (Moore *et al.* 1991). During the non-breeding period, band recoveries show some birds may travel up to 500km from their breeding territory (Darby and Seddon 1990). Their diet consists largely of juvenile fish, for which 95% is dominated by only seven species. These include sprat (*Sprattus antipodum*), red cod (*Pseudophycis bachus*), silverside (*Argentina elongata*), blue cod (*Parapercis colias*), ahuru (*Auchenoceros punctatus*), opal fish (*Hemeroceroetes monopterygius*) and squid (*Nootodarus sloanii*) (Van Heezik 1990), although other creatures such as krill have also been found as prey items (Moore *et al.* 1991). Squid represent the least nutritious prey species and its predominance in the diet has been used as an indicator of feed shortage to explain seasons of poor reproductive success (Van Heezik 1990). Although prey species of yellow-eyed penguins and commercial fisheries do not overlap for mainland penguins, the impact of fishery by-catch of yellow-eyed penguins in gill-nets remains a significant and ongoing problem (Moore and Wakelin 1997; Darby and Dawson 2000).

Adult yellow-eyed penguins return to land nightly, and remain on land for prolonged durations during nesting and moulting (McKinley 2001). Moulting occurs from February to April, during which time adults are unable to escape to the sea to feed or flee predators for a period of 3-4 weeks. In addition, hoiho have a specialised

requirement for visually isolated breeding sites and pairs may travel up to 1km inland to acquire a suitable nest (Seddon 1988; Darby and Seddon 1990). Thus while yellow-eyed penguins nest collectively, colonies are sparse and nest sites isolated, often at densities of 1- 5 nests per hectare (Darby and Seddon 1990). This unusual nesting behaviour is thought to be an evolutionary adaptation to breeding in warmer, temperate climates and demonstrates the utilisation of a once predator-free habitat (Anon. 1991). This dependence on terrestrial habitat has made yellow-eyed penguins increasingly vulnerable to habitat degradation and predation caused by human encroachment and the introduction of land-based predators.

Historically, nesting took place in coastal forest and shrubland (Marchant and Higgins 1990), but in response to the clearing of land for grazing many pairs can now be found nesting in grazed paddocks and the remnants of native forest (McKinley 2001). Destocking has been used to reduce farming impact, yet unstocked pasture can provide greater cover for predators and feral species than active farmland or native bush (Alterio *et al.* 1998). Remaining native habitat is often heavily degraded in addition to the presence of introduced species. Substantial habitat rehabilitation has taken place to restore nesting sites near native stands (Moore 2001). Predator control has formed a significant part of hoiho conservation and is used around a small proportion of colonies on the South Island. Mortalities on Stewart Island were initially attributed to cat predation, however recent evidence suggests predation on Stewart Island is of minimal impact (King 2007).

Threats and sustainability

Downward population pressure seems to have been acting on yellow-eyed penguins since the 1940's. Yellow-eyed penguins have not been able to rapidly adapt to dramatic alterations in their breeding environment, a fact compounded by their specialised behavioural traits and preference in habitat (Anon. 1991).
Threats identified in the initial recovery period included habitat loss through coastal clearing for grazing, habitat degradation by overgrazing and predation of adults and nestlings by stoats, ferrets, cats and dogs (Richdale 1957; McKinley 2001). Further threatening processes include periodic food shortage, climate change and occasional mortalities from commercial fishing (Van Heezik 1990; Anon. 1991; Peacock *et al.* 2000).

Disease has more recently been identified as a threatening factor for yellow-eyed penguins and could play an important role in population suppression. Documented infectious diseases in yellow-eyed penguins include *Pasteurella multocida*, aspergillosis and diphtheritic stomatitis (Duignan 2001; Alley *et al.* 2004). Aspergillosis was originally documented by Gill and Darby (1993) as a cause of death in a juvenile yellow-eyed penguin, and while rare in wild populations, it is a very significant disease in captive penguins. Diphtheritic stomatitis caused overall chick losses of 49% throughout the South Island in December 2002 with mortalities of up to 86% in some breeding areas (Alley *et al.* 2004).

Disease surveillance studies are limited to a serological survey of 18 yellow-eyed penguins on Campbell Island in 1988 which found no antibodies to infectious

bronchitis, reticuloendotheliosis, Newcastle disease, infectious laryngotracheitis, avian encephalomyelitis, infectious bursal disease, Marek's disease or fowlpox (de Lisle *et al.* 1990). It is important to note that these are all diseases of poultry with limited or no recorded pathogenicity to penguins.

Parasites affecting yellow-eyed penguins include the haemosporidia *Plasmodium relictum* (Fantham and Porter 1944; Laird 1950) and *Leucocytozoon* (Alley 2005), the coccidian *Eimeria*, the nematode *Contracaecum eudyptes* and the acanthocephalan *Corynosoma hannae*, without apparent measurable effects (McDonald 2003). The majority of data on the role of parasitism in yellow-eyed penguins has been acquired during seasons of relative environmental equilibrium so it is difficult to assess the effect of these organisms on the host in times of stress.

Plasmodium-like schizonts were identified in a yellow-eyed penguin submitted for post-mortem during chick deaths on the Otago Peninsula, but their role in the event was unknown (Alley 2001). *Graczyk et al*. (1995b) found 23-91% seroprevalence for *Plasmodium* antibody using ELISA in 6 populations of yellow-eyed penguins across New Zealand, however Sturrock and Tompkins (2007) found no evidence of *Plasmodium* in blood samples from 143 yellow-eyed penguins on the Otago peninsula using PCR. Malaria has not been conclusively linked to multiple mortality in free-ranging yellow-eyed penguins, but is considered a significant cause of death in captive penguins worldwide.

Leucocytozoon, a previously unrecognised haemoparasite in yellow-eyed penguins, was identified by Alley (2005) in chicks on Stewart and Codfish Islands in association

with ongoing chick mortalities. The underlying cause of these mortalities, which was previously attributed to predation, was not determined and is a significant focus of this thesis.

The accumulation of these factors and progressive population decline means that genetic sustainability is a central issue in future hoiho conservation. Triggs and Darby (1989) suggested regional genetic divisions of discrete breeding populations in the regions of the South Island, Auckland Islands and Campbell Island. They also estimated a minimum viable breeding population for each genetic grouping at 500 breeding pairs. Population estimates based on counts from 1988 to 1992 indicated a total of only 1780-2070 breeding pairs among 5930-6900 individuals (Anon. 1991). In this situation, mass mortalities have serious implications, with the capacity to reduce numbers to a point where incidental events may lead to loss of genetic diversity and destabilisation (Harwood and Hall 1990).

Mortality Events

Yellow-eyed penguin research was initially conducted by Richdale who surveyed local mainland populations extensively in the 1940's and 50's (Richdale 1957). Later studies in the 1980's focused on distribution, reproduction, predation, parenting, foraging and diet (Darby 1984; Seddon 1988).

Estimates of breeding populations are available for selected locations on the South Island using net searches, beach counts and observations (McKinley 2001), which has allowed the rough quantification of mortality events. Monitoring in less accessible

areas however, is infrequent at best, with the last reliable census for Campbell Island conducted in 1992 (Moore 1992b).

Between 1982 and 1996, the mainland yellow-eyed penguin population appeared unstable, with dramatic variations in the number of breeding pairs surveyed. The 1985 count of 600-620 pairs dropped to only 220 pairs in 1986-7, then recovered the following year to 400 pairs (Darby 1990). This spontaneous recovery was attributed to a significant proportion of the population not attempting to breed in 1986-7. In 1988, only 3 areas in the entire South Island had breeding areas containing more than 30 adult pairs (Darby and Seddon 1990).

A further decline occurred in 1989-90 with the loss of 30-60% of the adult breeding population at Otago peninsula, signifying a return to dramatic adult mortality. The following season only 190 breeding pairs remained on the South Island (Moore *et al.* 1991). Darby and Seddon (1990) suggested that populations in the Catlins and Southland areas had declined by 75% over the previous 40 years.

Offshore sites have also been affected, with populations at Campbell Island falling by at least 69% between 1987 and 1991 (Moore and Moffat 1990). These events represent a string of seasonal mortalities with several major incidences of poor breeding success and low adult survival since the mid 1980's (Moore *et al.* 1991). Furthermore, Van Heezik indicated in 1990 that the frequency of poor seasons may be increasing (Van Heezik 1990).

Multiple explanations for these declines have been proposed. The mortality event on the Otago coast during the breeding season and following moult of 1985-86 was reportedly due to a shortage of prey species (Van Heezik 1990), with suggested links to El Nino events (Moore *et al.* 1991). The following decline in breeding numbers in 1987-88 was also attributed to starvation with low food abundance (Darby and Seddon 1990). In this season the Catlins and South-East Otago registered mean losses of up to 80% (Anon. 1991). In the 1989-1990 season, when neurological signs were first observed (Duignan 2001), body condition was used to rule out starvation and the primary cause of death could not be established (Moore *et al.* 1991). In later work, Moore suggested that these individual crises are part of long-term fluctuations, resulting from the poorly understood interplay between human activity and environmental variation (Moore 2001).

With interest in yellow-eyed penguin conservation stemming from the early 1980's, a significant effort has been invested in minimising known threats to their survival. In 1990, after the magnitude of the previous season's mortality was realised, chicks were captured and captive reared to encourage adult survival. While this exercise may have prevented adult mortality, only 3 of 194 chicks raised were known to have survived to the following season (Darby and Paterson 1991). During this time population decline has not been limited to yellow-eyed penguins, as dramatic declines in the number of erect-crested and rockhopper penguins were also described (Duignan 2001).

At a regional level, population counts on Stewart Island in 2003 revealed a population in decline compared to surrounding islands (Darby 2003). Early chick deaths had

reduced reproductive success to 21-33% without significant predation (King 2007). Eight yellow-eyed penguin chicks from Stewart and Codfish Islands showed histological evidence of leucocytozoonosis and presented with anaemia, weight loss, sudden death and severe subcutaneous ecchymotic haemorrhage (Alley 2005). As a result of this association with mortality and morbidity in chicks, *Leucocytozoon* was considered a significant factor requiring further investigation.

Haemoparasites (Apicomplexa: Haemosporidia)

Avian haemosporidia are found worldwide, and consist of 3 genera, *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, which are present in over 70% of species. Extensive knowledge has been acquired from studies of *Plasmodium* spp. as a model for human malaria, however little is known about other haemosporidia, which form the majority of wild avian infections (Desser and Bennett 1993). Many haemosporidial infections are considered incidental in parasite-adapted hosts, however their presence can represent a significant burden on host activity and immunity, and in heavy or combined infections can directly cause morbidity and death (Fallis *et al.* 1974).

Avian haemoparasitism has been shown to reduce reproductive success and depress species resilience to change (Jones and Shellam 1999b; Merino *et al.* 2000). Given the right conditions, some haemoparasites are capable of shaping entire ecosystems and threatening endangered species (Kilpatrick 2006). The introduction of *Plasmodium* to the Hawaiian Islands contributed to multiple avian extinctions, such as

the Kamao (*Myadestes myadestinus*), and permanently altered the ecology of avian species which remained (Kilpatrick 2006).

Little is known about the physiological, behavioural and ecological cost of haemoparasites in wild hosts. Extrapolation is difficult from studies based on laboratory models and in domestic species, which fail to account for biological or environmental variability such as stress, age, genetics, immunocompetence or nutrition (Atkinson and Van Riper III 1991).

There appears to be variable susceptibility to avian haematosporidia between the avian family groups and this may involve behaviour, habitat, vector availability, climate, host-specificity and co-evolution of parasites (Atkinson and Van Riper III 1991). *Haemoproteus* and *Leucocytozoon* species appear relatively host specific (Fallis *et al.* 1974; Atkinson and Van Riper III 1991; Bennett 1994), while *Plasmodium* species cover a broader host range extending across families (Bennett *et al.* 1982).

Haemoparasitism in Penguins

Penguins are susceptible to both *Plasmodium* and *Leucocytozoon*, however *Haemoproteus* infections have not been recorded in the wild or in captivity (Jones and Shellam 1999). The majority of free-living penguins are not infected with haematozoa within their natural range (Peirce *et al.* 2005). Penguins breeding in the Antarctic and Subantarctic are virtually free of haemoparasites, presumably due to limitations in vector availability (Jones and Shellam 1999b). Those species which

have evolved in more temperate climates, such as yellow-eyed, Fiordland crested (*Eudyptes pachyrhyncus*), African (*Spheniscus demersus*), rockhopper (*Eudyptes* chrysocome) and little (*Eudytpula minor*) penguins, are far more likely to contact haemoparasites amid the vectors and reservoirs of other haemoparasitised species (Jones and Shellam 1999b).

In captive penguins, *Plasmodium* infection is considered a worldwide disease risk, in which exposure of naïve hosts and activation of latent infections can occur (Fix *et al.* 1988). The stress of transport, handling or captivity can revive previously undetected parasitaemias and cause mortalities (Cranfield *et al.* 1994; Graczyk *et al.* 1995a). *Plasmodium* dominates the literature in captive penguin disease with the lower prevalence of *Leucocytozoon* as a cause of captive penguin mortality most likely resulting from a higher host-specificity, differing vector distribution or lower pathogenicity. *Haemoproteus* is not known to cause significant disease in other avian species, except to exacerbate the effects of concurrent infections (Evans and Otter 1998).

The low prevalence and disease intensity of haemoparasitism in wild species is thought to relate to ecological traits, with prolonged aquatic periods limiting host exposure. Captive penguins are denied this escape and suffer heavy infections with high morbidity and mortality (Jones and Shellam 1999b). Temperate nesting species with short, inland foraging strategies as seen with yellow-eyed penguins are also exposed frequently to vectors and promote the completion of the protozoal life-cycle (Croxall and Davis 1999). Individual immunity, species susceptibility, and pressures

causing immune depression will also play significant roles in parasite prevalence and the expression of disease.

Leucocytozoon: the parasite in question

Leucocytozoon is an obligate, intracellular protozoal parasite of birds, which reproduces within blood and tissues, destroying the host cells. Many *Leucocytozoon* species are considered as incidental parasites, however some species, such as *L. simondi and L. caulleryi*, develop significant reproductive stages in tissues and can be highly pathogenic (Desser and Bennett 1993).

Infection with *Leucocytozoon* is common to avian families worldwide, yet the impact of *Leucocytozoon* in wild species is not well known (Atkinson and Van Riper III 1991). The fundamentals of what is known about *Leucocytozoon* are derived from laboratory studies with production birds and from wild bird post-mortems (Atkinson and Van Riper III 1991). In some species, heavy infections, or infections by multiple haematozoa species, are capable of causing severe anaemia, tissue inflammation and death (Herman *et al.* 1975; Evans *et al.* 1998), while evidence in other studies suggests the true impact lies in decreased reproductive performance (Merino *et al.* 2000; Dunbar *et al.* 2003).

In endangered species, decreased reproductive success can be as significant to decline as actual mortality events. Suppression of *Leucocytozoon* through medication in free-living blue tits (*Parus caeruleus*) caused improved reproductive success and post-breeding condition, demonstrating the negative effect of haemoparasitism on parental

working capacity (Merino *et al.* 2000). Control animals affected by *Leucocytozoon* suffered reduced fledgling success and females showed parasitism-dependent loss of condition (Merino *et al.* 2000).

Leucocytozoon causes serious disease in domestic and wild birds in many parts of the world (Atkinson and Van Riper III 1991). Epizootic infections with *L. simondi* have caused seasonal mass-mortality of Canada geese (Fallis *et al.* 1974; Herman *et al.* 1975; Atkinson and Van Riper III 1991), while *L. caulleryi* has threatened waterfowl (Fallis and Desser 1977), and caused mortality and decreased production in poultry across Asia (Morii 1992). The combination of large springtime populations of black flies and susceptible goslings in Canada created periods of intense *Leucocytozoon* transmission and fatal infections (Herman *et al.* 1975). These epizootics appeared to run in 4-year cycles, not unlike the pattern of adult mortality seen in yellow-eyed penguins.

Similar epizootic events are associated with the transportation of naïve hosts into endemic areas, or the introduction of infected vectors into naïve populations (Atkinson and Van Riper III 1991). Such scenarios are of great importance in the management of endangered or threatened species whether in translocation or re-introduction of captive species (Atkinson and Van Riper III 1991). Localised outbreaks of *Plasmodium* in captive penguins have occurred in a variety of zoos due to transportation or changes in management (Fix *et al.* 1988; Desser and Bennett 1993; Peirce *et al.* 2005).

Leucocytozoon has been identified in three free-ranging New Zealand species. *L. tawaki* was found in Fiordland crested penguins, *L. fringillinarum* in an introduced chaffinch (Laird 1950), and an unnamed *Leucocytozoon* sp. in yellow-eyed penguins (Alley 2005). *L. tawaki* has also been identified in blood smears from wild-caught, captive Macaroni penguins in the United Kingdom which suffered deaths due to *Plasmodium* infection (Peirce *et al.* 2005), and an African penguin with a combined *Leucocytozoon* and *Plasmodium* infection observed during an oil spill in South Africa (Earle *et al.* 1992). Successful vector transfer of *L. tawaki* from a Fiordland crested to a Little penguin has been experimentally demonstrated, although the resulting pathology was not described (Allison *et al.* 1978).

Prevalence

Prevalence of haemoparasites and their effects on the ecology of the host species are inextricably linked, and may differ greatly between host species and location (Fallis *et al.* 1974; Desser and Bennett 1993). Prevalence and disease intensity can provide indications of host susceptibility, availability of suitable vectors, and preference of vectors for certain species (Desser and Bennett 1993). On a global scale, the prevalence and distribution of avian blood parasites are dynamic, affected by any forces influencing distribution, spread, and density of the intermediate host (Desser and Bennet 1993). In the present case, the population fluctuation of yellow-eyed penguins may add a further dynamic to both prevalence and intensity of infection. As populations become smaller, more condensed and more exposed, the prevalence of infection spread by endemic vectors is likely to be increased.

Mountainous areas with numerous streams provide optimal breeding environments for simuliids, and will most likely contribute to a higher prevalence of *Leucocytozoon* whereas low, marshy terrain more suitable for mosquitoes will be more likely to contain *Plasmodium* (Desser and Bennett 1993). Accordingly, the occurance of *Leucocytozoon* in species in Fiordland and Stewart Island represents areas were simuliid breeding environments are readily available.

Clinical signs

Leucocytozoonosis causes anaemia, dyspnea, lethargy, inappetence and death (Desser and Bennett 1993; Greiner and Ritchie 1994). Central nervous signs have been documented (Raidal and Jaensch 2000) which may be caused by schizont formation and capillary blockage within the brain. Biliverdinuria often develops due to erythrocyte destruction and hepatic inflammation and necrosis. Gross pathology includes hepatomegaly, splenomegaly, pulmonary congestion, pericardial effusion and haemorrhages (Desser and Bennett 1993; Greiner and Ritchie 1994; Aiello and Mays 1998).

Leucocytozoon is considered highly pathogenic in Anseriformes (ducks and other waterfowl) and Galliformes (chickens and turkeys) (Greiner and Ritchie 1994), with mortalities occurring mostly in young or underweight birds (Desser and Bennett 1993). Fatal infections in other species have been described sporadically involving budgerigars (Greiner and Ritchie 1994), weavers (Garnham 1950) and pigeons (Peirce *et al*. 1997).

Morbidity and mortality are thought to be associated with megaloschizont formation (Fallis *et al.* 1974; Desser and Bennett 1993) and an anaemia caused by the release of anti-erythrocytic factors (Kocan 1968). Thus, clinical signs may be referable to affected organs, such as the brain, liver and kidney.

The life-cycle: general

The successful completion of the haemosporidial life-cycle is reliant on a number of critical steps; an appropriate and available vector possessing the correct feeding preference; a critical density of hosts; and an ecosystem favourable to the vector and the parasite within the host (Jones and Shellam 1999b). The feeding preference of the parasite will influence the ability of infection to spread across species boundaries, with generalists such as *Plasmodium* becoming far more widespread than more specialised haemoparasites such as *Leucocytozoon* (Atkinson and Van Riper III 1991).

While the Apicomplexa share a close evolutionary relationship, the three genera demonstrate a wide variation in preferred ecology and life-cycle (Beadell and Fleischer 2005). For example, the vectors of each are quite independent; *Plasmodium* in culicine mosquitoes, *Haemoproteus* in hippoboscid and ceratopogonid flies and *Leucocytozoon* in simulid flies (Atkinson and Van Ripper III 1991). Some variation to this pattern has been observed, such as the transmission of *L. caulleryi* by *Culicoides arakawae* (Akiba 1960).

The clinical phases of all Apicomplexa are similar, undergoing a prepatent phase of initial schizogony in tissues, an acute phase of haematogenous spread and erythrocytic involvement, a crisis phase at peak parasitaemia and physiological stress, and finally a latent phase with immunological control, but not removal, of infection (Atkinson and Van Riper III 1991). Once established, chronic infections may remain undetectable in the blood until periods of stress or hormonal activation occur, such as seasonal relapse (Atkinson and Van Riper III 1991).

The life-cycle of *Leucocytozoon*

The life-cycle of *Leucocytozoon* was first established in the early 1930's in waterfowl (O'Roke 1930; 1931) and turkeys (Skidmore 1931) with recognition of both host and vector stages (Parker *et al.* 2006). Despite ongoing research, very few complete life-cycles are known (Desser and Bennett 1993). Generally speaking, the Apicomplexa have simple life-cycles of asexual reproduction in host and sexual reproduction in vector (Fallis *et al.* 1977). At a finer level its development can seem far more complex.

Sporozoites, the infective stage, access the host via the bite of the vector, a simulid fly. They are carried to the host's liver via the bloodstream where they invade hepatocytes and endothelial cells and develop as primary schizonts. Schizonts expand within the host cell, undergoing asexual reproduction known as schizogony, wherein multiple uninucleate, membrane-lined merozoites are formed by repeated invaginations of the internal parasite plasma membrane. Schizogony takes place within the host cell membrane and does not cause inflammation, although heavy

infections can result in dilation and congestion of sinusoids. In some species, merozoites are contained in discrete packets called cytomeres prior to release. Schizonts mature and rupture the host cell, releasing merozoites that are either phagocytosed or infect circulating leucocytes and erythrocytes (Fallis *et al.* 1974; Desser and Bennett 1993).

Upon entering the cytoplasm of circulating cells, merozoites develop into gametocytes which can be seen in peripheral smears. No further development takes place from this stage within the avian host. The sexual stage of gametogony occurs within the midgut of the vector after a blood meal containing gametocytes, followed by sporogony in the coelom. At the erythrocyte level, *Plasmodium* exhibits erythrocytic schizogony, which can be used to differentiate *Plasmodium* infections from *Leucocytozoon* or *Haemoproteus* (Atkinson and Van Riper III 1991). This difference also ensures that while *Plasmodium* can be spread by blood inoculation directly between hosts, the vector stage of *Leucocytozoon* is essential for complete development (Desser and Bennett 1993).

Parasitised erythrocytes may be removed from circulation in the spleen, causing anaemia of varying degrees. Damage may be also be caused by the release of anti-erythrocyte factors producing anaemia in acute infections (Kocan 1968; Atkinson and Van Riper III 1991). *Leucocytozoon* also has an affinity for leucocytes and will parasitise monocytes, lymphocytes and endothelial cells. Excessive numbers of circulating gametocytes may produce direct pathology, causing pneumonia, pulmonary congestion and embolic infarctions in infected turkeys (Siccardi *et al.*

1974). Other pathology includes splenomegaly, lymphocytic infiltration of the liver and heart, hepatic necrosis and haemosiderosis (Atkinson and Van Riper III 1991). Fallis *et al.* (1974) suggested that two types of schizogonic cycle exist, exemplified by *L. simondi*, and *L. dubreuili* respectively. *L. simondi* features primary schizogony in the liver with schizonts up to 45 µm diameter, which discharge merozoites after 4-6 days of development in the duck. Merozoites develop into round gametocytes in circulating cells, but incompletely divided merozoites, called syncytia, are phagocytosed by macrophages or endothelial cells to create megaloschizonts. Megaloschizonts develop in a similar fashion to primary schizonts, but grow to 200 µm over 4 days, causing marked hypertrophy of the host nucleus and cytoplasm. On rupture, they release large numbers of merozoites which develop into elongate gametocytes. Maximum parasitaemia in ducks occurred at 10-12 days and infections can be retained for at least 2 years (Fallis *et al.* 1974).

Megaloschizonts in this first type of cycle seem to be the main source of pathogenicity, becoming encapsulated in fibrous tissue to eventually die and calcify before causing inflammation on rupture. Those species that do not form megaloschizonts do not seem to express the same pathogenicity (Atkinson and Van Riper III 1991).

In comparison, *L. dubreuili* and *L. fringillarum* develop primary schizonts in hepatocytes and renal epithelial cells over 3-4 days, but no megaloschizont formation occurs. Round gametocytes are observed without the production of elongate gametocytes. This distinction has led to the association of elongate gametocytes with megaloschizonts and pathology, and round gametocytes with simple schizonts without

tissue pathology.

Leucocytozoon structure

Leucocytozoon was first described in an owl by Danilewsky in 1884, and since that time over 67 species have been identified in the genus (Levine 1988). Species are morphologically similar and usually differentiated by macrogametocyte morphology and the identification of the host (Fallis *et al.* 1974). The latter method can become confusing as multiple species have been found in the same host and numerous synonyms have arisen (Desser and Bennett 1993). Further complications arise when gametocyte morphology changes during the course of infection.

The following is an outline of the structure of *Leucocytozoon* during its various phases and some finer details of development. Diverse patterns of schizogony and sporogony have been reported among the *Leucocytozoon* species, and the character of the endogenous development, where it has been studied, varies widely. Most comparisons are made with *L. simondi* or *L. dubreuili* so reference to these has been included.

Hepatic Schizonts

Schizonts represent the primary tissue phase, and occupy the cytoplasm of the host cell within a thin membrane-bound vacuole. The host cell is generally distorted by the growth of the parasite until its original definition is lost (Desser 1972). In the majority of *Leucocytozoon spp*. schizogony occurs in the liver. Others, such as *L.*

caulleryi in chickens, undergo primary schizogony in the endothelial cells of visceral tissue (Desser and Bennett 1993).

Schizonts contain multiple electron-dense, multinucleate cytomeres bound by trilaminar plasma membranes within the parasite cytoplasm containing multiple ribosomes (Desser 1972). Repeated invagination of the cytomere produces merozoites from each of the many nuclei which are lined by a single plasma membrane (Desser 1972; Fallis *et al.* 1974). The parasite nucleus is contained within a trilaminar membrane and features electron dense, peripherally arranged chromatin (Desser 1972). The development of merozoites is not synchronous, such that a range of sizes and stages of maturation are observed within the same tissues (Desser and Bennett 1993).

Merozoites

Merozoites of *Leucocytozoon* are also contained within a trilaminar plasma membrane and are markedly shorter and stubbier than those of *Plasmodium* or *Haemoproteus* (Desser and Allison 1979). They are initially observed within the cytomeres of primary schizonts, forming by repeated invagination. Merozoites developing within megaloschizonts multiply by a combination of cytomeric invagination and nuclear fission. They may be differentiated into male (micromerozoites) and female (macromerozoites) by the high concentration of ribosomes and electron-dense material in the latter (Desser and Bennett 1993).

On electron microscopy, merozoites contain a large central nucleus, and a mitochondrion with vesicular cristae (Desser 1972). Some species demonstrate one or

two prominent refractile or ‘paranuclear’ bodies which are electron dense and homogeneous (Scholtyseck 1979). Organelles include paired electron-dense rhoptries and micronemes associated with 3 apical rings (Fallis *et al.* 1974). Membrane-bound ovoid bodies containing non-descript structures which may represent protein granules contribute to the background within the cytoplasm (Scholtyseck 1979).

Occasionally schizonts release incompletely differentiated merozoites called syncytia. These consist of dense, paired nuclei coupled within a single plasma membrane, and are thought to be responsible for the formation of megaloschizonts (Desser and Bennett 1993). Desser and Bennett (1993) postulated that syncytia may be abnormal merozoites resulting from premature rupture, or evidence of a poorly host-adapted parasite.

Gametocytes

Gametocytes are parasitised erythrocytes and leucocytes which have been invaded by merozoites. The male (microgametocyte) has a larger, more diffuse nucleus and more pale appearance than the female (macrogameocyte). Macrogametocytes are more numerous and contain dense nuclei and extensive electron-dense material within the cytoplasm. They produce pseudopigment known as volutin, made of metachromatic inclusion bodies which contain a high proportion of RNA, but lack the refractile pigment deposition seen in erythrocytic phases of other Apicomplexa (Fallis *et al.* 1974; Atkinson and Van Riper III 1991; Desser and Bennett 1993).

The time from inoculation to the appearance of gametocytes in the blood (the prepatent period) ranges from 4 -14 days and is dependent on host and parasite species. This period appears to be more prolonged in light infections (Fallis *et al.* 1974).

Many *Leucocytozoon* species produce two types of gametocytes. The first appear as round gametocytes in round cells with laterally displaced nuclei, and the second, elongate gametocytes, are round, oval or elliptical parasites within an elongated or fusiform cell. The nucleus of the host may be distorted, extending the length of the parasite, or unchanged. Elongate gametocytes are more common in non-passerine birds. While gametocyte morphology is an important means of species identification, some *Leucocytozoon* spp. alter the production of round or elongated cells at different stages of infection. This variation has led to significant confusion in the literature regarding the identification of new *Leucocytozoon* species (Fallis *et al.* 1974; Desser and Bennett 1993).

Fallis *et al.* (1974) proposed that round gametocytes develop from primary schizonts and elongate gametocytes from megaloschizonts, the latter exclusively in leucocytes. A notable exception to this classification is *L. sakharoffi* which represents a small but growing number of *Leucocytozoon* spp. that feature megaloschizonts and exclusively round gametocytes, thus the link between gametocyte structure and schizont structure remains poorly understood (Fallis *et al.* 1974).

Sporogony (Vector stage)

The sporogonic cycle is initiated within the midgut of the vector where the gametocyte undergoes exflagellation, exiting the host cell. The stimulus is the alteration in oxygen tension between the avian and insect hosts, and the entire process requires approximately one week to complete at 20°C. Different species appear to have different temperature preferences, with cold-adapted species developing faster in cooler rather than hotter conditions (Desser and Bennett 1993).

Nuclear chromatin from the microgametocyte condenses to form eight separate, motile microgametes which seek out macrogametes formed in a similar way. Sexual reproduction involves fertilisation of the macrogamete, leading to meiotic division and transformation into an elongated, motile ookinete. This process occurs within a few hours. The ookinete is capable of penetrating the gut lining and becomes an oocyst, from which sporozoites will be released into the coelom to migrate to and penetrate the salivary glands of the vector (Desser and Bennett 1993).

Megaloschizont

Megaloschizonts are coated by a thick external capsule consisting of outer fibrous and inner filamentous layers bordering the host membrane. Numerous cytomeres, similar to those contained in primary schizonts, develop from the periphery, containing multiple merozoites. The parasite is thought to stimulate the host cell, causing hypertrophy with increases in nuclear chromatin and cytoplasmic mitochondria and endoplasmic reticulum. The parasite is bounded by a double membrane, and eventually displaces and compresses the contents of the host cell (Fallis *et al.* 1974).

Leucocytozoon in penguins

The first *Leucocytozoon* infections identified in yellow-eyed penguins consisted of spherical cysts up to 80μm diameter, containing several compartments of basophilic, granular, bradyzoite-like structures. Inflammation was noted around degenerate cysts in multiple tissues, including the spleen which was often hyperplastic with evidence of extramedullary haematopoesis (Alley 2005).

Leucocytozoon was first described in penguins by Fallis *et al.* (1976) identifying *L. tawaki* in the Fiordland crested penguin, with later studies exploring the life-cycle and development in the primary vector, *Austrosimulium ungulatum* (Fallis *et al.* 1976; Allison *et al.* 1978; Desser and Allison 1979). No further descriptions of the endogenous life-cycle have been made since then.

In wild penguins *L. tawaki* is not known to be pathogenic, however, it may have the potential to be pathogenic in some penguins. Given the presence of Fiordland crested penguins and *Austrosimulium* flies in yellow-eyed penguin breeding areas on Stewart Island this species is of intense interest.

The primary schizonts of *L. tawaki* in Fiordland crested penguins occur in the liver, kidney and other tissues. Both schizonts and erythrocytic gametocytes were present in 4-6 week old chicks. Hepatic schizonts were roughly spherical, 17-22 μm diameter, and contained merozoites while renal schizonts were spherical to ovoid and measured up to 50 μm in diameter. Adult specimens contained renal, but not hepatic

schizonts, and a single 35x40 μm endothelial schizont was found in the spleen (Allison *et al.* 1978)

The daily feeding peak of *Austrosimulium ungulatum* during spring coincided with the highest daily temperatures, suggesting temperature plays a role in levels of transmission (Desser and Allison 1979). Peak host parasitaemias have also been noted during daylight hours, coinciding with the prominent feeding periods of the vector (Bennett and Fallis 1960). Similar to simuliids carrying *L. simondi* from ducks, those flies feeding on lightly infected chicks during the September to November period had longer survival times than those feeding on heavily infected individuals, suggesting there are negative effects of parasitism for vectors (Allison *et al.* 1978).

Fallis *et al.* (1974) proposed that chicks are not likely to be infected until 2-3 weeks old when they move away from their parents. Avian hosts infected as chicks, will probably harbour the parasite for the rest of their lives (Fallis *et al.* 1974). Within the Fiordland study group, a typical pattern of heavy parasitaemias in chicks and lighter burdens in mature birds were seen. Allison *et al.* (1978) also noted that heavy infections might render young chicks more susceptible to secondary infection and more sensitive to stressors such as weather. Timing of transmission is therefore a critical factor in outbreaks, as nestling birds are most susceptible and will incur the greatest morbidity and mortality (Atkinson and Van Riper III 1991).

It is essential that studies of parasite effects on particular species are based on data extracted from that species, rather than extrapolated from others. The life-cycles of

some species described by Fallis *et al.* (1974) are not consistent with megaloschizont formation, which is seen in the life-cycles of *L. tawaki* and *L. simondi* (Allison *et al*. 1978). Allison *et al*. (1978) suggested that while the schizogony of *L. tawaki* was similar to other species studied, with primary schizogony in hepatocytes and secondary schizonts in the proximal renal tubules, the observation of a splenic endothelial schizont was not characteristic of this pattern. Allison *et al*. (1978) went on to describe that sporogonic development in *L. tawaki* was in fact very similar to *L. dubreuili* in its capacity for extensive expansion and high sporozoite production. Investigation of the morphology and life-cycle of *Leucocytozoon* in yellow-eyed penguins is required to understand its relationship with *L. tawaki* and other *Leucocytozoon* spp.

Vectors

Transfer of *Leucocytozoon* from host to host is accomplished by simuliid flies, commonly known as biting black flies. Only *L. caulleryi,* of the subgenus *Akiba*, is transmitted by several species of *Culicoides* biting midges (Desser and Bennett 1993). New Zealand black flies are represented by the genus *Austrosimulium,* including *A. austalense* on the mainland and *A. ungulatum* on Stewart Island and higher latitudes of the South Islands (Allison *et* al. 1978). It is possible that suitable vectors for the spread of *Leucocytozoon* may be present in the Subantarctic (pers com. Sandy King; Duignan 2001).

While vectors are vital for *Leucocytozoon* development and distribution, it is the host that sustains the protozoal infection between seasons. Infected vertebrates must be

present to amplify infections and continue the host-vector-host cycle. The actual mechanism of this persistence, however, is poorly understood.

Diagnosis

Initial testing for haemoparasitism relied on identification and morphological examination of erythrocytic stages in blood smears. However, studies have shown that the prevalence of parasitaemia may not reflect true prevalence of infection in a population (Fix *et al*. 1988; Graczyk *et al*. 1995c). While blood smears still have a role to play in disease investigation, prevalence studies now rely on PCR, ELISA and latex agglutination techniques for identification in addition to traditional blood smear morphology (Graczyk *et al.* 1995c; Freed and Cann 2003; Hellgren *et al.* 2004; Ito and Gotanda 2005; Swinnerton *et al.* 2005; Sturrock and Tompkins 2007).

Molecular techniques can detect infections with far greater sensitivity than can traditional light microscopy, but require significantly more equipment and technical expertise (Freed and Cann 2003). Due to its complexity, there is often considerable variation between the results of different laboratories, and between different studies (Freed and Cann 2003). This can be reduced, but not eliminated, by applying standardised laboratory protocols. DNA sequencing is important in identifying the actual causative agent of disease. A study of *Plasmodium* by McConkey *et al*. (1996) found that the use of PCR without sequencing could confuse pathogenic and non-pathogenic species when used as a screening tool. When used in combination with PCR, sequencing formed an important part in our study to identify the relationship of *Leucocytozoon* sp. in yellow-eyed and Fiordland crested penguins.

Restriction-based assays have been produced to detect and identify multiple haemosporidian species (Beadell and Fleischer 2005). More specific testing for a range of species has been developed, such as recombinant antigen-based latex agglutination against *Leucocytozoon* (Ito and Gotanda 2005), however the application of these is often limited to individual host species.

Species susceptibility

Haemoparasite prevalence is not a species-specific constant, but rather a fluctuation dependent on breeding cycle (seasonal parasitaemias), environment and population factors (Tella *et al*. 1999). This must be remembered when comparing between populations and subpopulations where environments or ecosystems may vary.

Further, sedentary species such as yellow-eyed penguins are more likely to be parasitised than migratory species, and individuals of species breeding in forested habitats are far more parasitised than those in more open habitats (Tella *et al*. 1999). This is most likely an expression of higher vector prevalence in heavily vegetated terrain. It has been suggested that as a result, species with low immunocompetence against parasitaemia may evolve with a preference for more open habitats where prevalence is low (Tella *et al*. 1999). Assumptions should not be made, however, in interpreting species immunocompetence solely on the basis of their nesting habits.

Research to date suggests that immuno-naïve chicks are most susceptible to infection, and that rate of transmission, enhanced by temperature, rainfall and vectors, is a primary factor in morbidity and mortality in seasonal outbreaks. Furthermore, it is

evident that the study of *Leucocytozoon* in wildlife would be complemented by the investigation of vectors and host physiology.

The chronic form of *Leucocytozoon* infection is poorly understood, but it is thought that stress has a primary role in relapses (Atkinson and Van Riper III 1991). Cranfield *et al*. (1994) proposed that malarial recrudescence is due to the persistence of infected erythrocytes in deep vascular sites, or in dormant sporozoites in endothelial tissues, and that erythrocyte invasion is associated with immunosuppression. As *Leucocytozoon* has a more marked tissue phase than other haemoparasites, recrudescence after the cessation of immune response or treatment is also more likely.

Climate trends in New Zealand have been toward warmer minimum temperatures which may allow increasing vector reproductive ability, or greater distribution (Salinger and Griffiths 2001). Peacock *et al*. (2000) found that fledgling success was in fact higher during slightly cooler seasons. They also suggested that long term climate change in general, rather than El Nino-Southern Oscillation events specifically, was more likely to be among the underlying causes of yellow-eyed penguin population decline (Peacock *et al*. 2000).

Significance of *Leucocytozoon* research

As reduced predation and habitat restoration are achieved, attention must be directed to more insidious threats affecting the health and stability of wild penguin populations (Jones and Shellam 1999b). *Leucocytozoon* represents such a threat in a rare and

already unstable species. While data is lacking, the risk to both penguins and translocated native species cannot be quantified or fully understood.

It is apparent that heavily parasitised individuals may quickly become malnourished, although cause and effects are difficult to separate, and malnutrition is an important factor in immunodeficiency and susceptibility to disease (Gershwin *et al*. 1985). Diseased animals are also more susceptible to mortality in other forms, such as predation and starvation (Hudson *et al*. 1992).

Should *Leucocytozoon* prove to have a significant role in yellow-eyed penguin mortalities, its management may allow increased reproductive success and population growth. Its identification will provide information on the interactions of yellow-eyed and Fiordland crested penguins, and insight into future treatment and prevention measures.

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Chapter 2

Prevalence of *Leucocytozoon* in the endangered yellow-eyed penguin, *Megadyptes antipodes*

For submission to the *Journal of Wildlife Disease*

Abstract

Yellow-eyed penguins on Stewart Island were identified with a novel *Leucocytozoon* sp. in association with a high regional incidence of chick mortality (n=32, 100% mortality). A PCR survey of blood from 119 birds from 5 distinct nesting areas on the South Island (n=95), Stewart (n=12) and Codfish Island (n=12) found *Leucocytozoon* infections only on Stewart Island (n=10, 83%). *Leucocytozoon* sp. DNA sequences taken from blood and tissues were similar between yellow-eyed penguin adults (n=10) and chicks (n=7), but different to *Leucocytozoon* sp. DNA obtained from Fiordland crested penguins (n=2) whose nesting range overlaps at Stewart Island. These findings suggest that yellow-eyed penguins on Stewart Island suffering severe chick mortality are infected with a regionally isolated, host specific *Leucocytozoon* sp.

Introduction

The endangered yellow-eyed penguin, an endemic species of southern New Zealand, has suffered major population declines and periodic mass mortality. These events, with losses of up to 60% of the breeding adults in some areas, may have a significant

impact on the survival of the species (Darby and Seddon 1990). The cause of these mortality events, and the possible role of infectious disease, has never been conclusively determined (Duignan 2001; McKinley 2001; Darby 2003; Alley *et al*. 2004). Previous declines have been attributed to avian malaria, food shortage and marine biotoxins, but without confirmation of the suspect agent (Darby and Seddon 1990; Van Heezik 1990; Moore *et al*. 1991).

The total estimated breeding population of yellow-eyed penguins is around 2000 pairs (McKinley 2001). They nest in sparse colonies and are dependent on their limited terrestrial habitat to breed (Darby and Seddon 1990). The main nesting areas, as shown in Figure 1, are Stewart Island (est 170-320 pairs), Codfish Island (50-80 pairs) (Darby 2003), the South Island (300-320 pairs), and the subantarctic Auckland and Campbell Islands (1010-1170 pairs) (McKinley 2001). The South Island features three main colonies at Oamaru, Otago Peninsula and the Catlin coast.

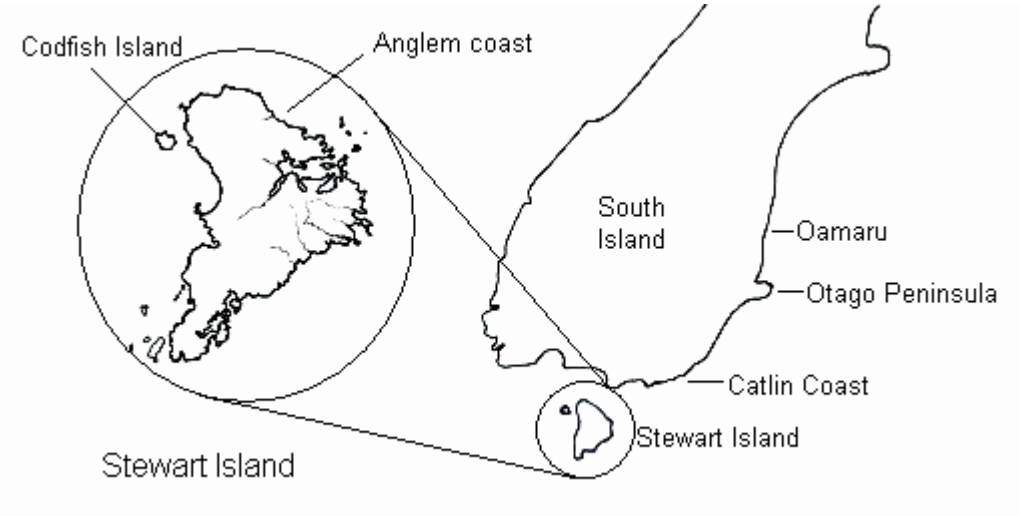

Fig 1. Major yellow-eyed penguin nesting sites on the South Island, Stewart and Codfish Islands used in this study.

Stewart Island is the largest nesting region outside of the subantarctic, and features high chick mortality and low reproductive success compared to other nesting sites (King 2007). Examination of chick mortalities in 2005 on Stewart and Codfish Islands identified eight cases of leucocytozoonosis, a previously unreported disease of yellow-eyed penguins (Alley 2005).

Leucocytozoon is a host-specific haemoparasite of the Apicomplexa phylum and is found in birds worldwide. *Leucocytozoon* has been identified in Fiordland crested penguins (*Eudyptes pachyrynchus*) in New Zealand (Fallis *et al*. 1976), whose range overlaps with yellow-eyed penguins at Stewart Island, and in captive Macaroni (*Eudyptes chrysolophus*) (Peirce *et al*. 2005) and African (*Sphenisus demersus*) penguins (Earle *et al*. 1993).

Leucocytozoon affects circulating leucocytes and erythrocytes as well as tissue macrophages and endothelial cells, where it creates large tissue schizonts up to 700 µm (Atkinson and Van Riper III 1991). Some *Leucocytozoon spp*. are pathogenic, causing mortality or reduced fertility and growth . Epizootic infections with *Leucocytozoon simondi* have caused seasonal mass-mortality of Canada swans (Fallis *et al*. 1974; Herman *et al*. 1975; Atkinson and Van Riper III 1991), while *L. caulleryi* has threatened waterfowl (Fallis and Desser 1977), and caused mortality and decreased production in poultry across Asia (Morii 1992). In addition, Merino *et al.* (2000) demonstrated detrimental effects of *Leucocytozoon* on host reproductive success and condition in blue tits.

Other diseases identified in yellow-eyed penguins include aspergillosis, malaria (*Plasmodium*), and the syndrome of diphtheritic stomatitis, which caused major chick mortality in the Catlin coast in 2002 (Alley *et al*. 2004). A single case of mortality due to *Plasmodium*, a related Apicomplexa to *Leucocytozoon* spp., was diagnosed in a yellow-eyed penguin from the Otago peninsula in 2004 (Alley *et al*. 2004). However, a recent study failed to identify *Plasmodium* spp. in 143 yellow-eyed penguins from Otago Peninsula (Sturrock and Tompkins 2008), despite historical studies finding high seroprevalence (Graczyk *et al*. 1995a; 1995b).

The aim of this paper is to investigate the role of *Leucocytozoon* in chick mortalities, and establish the prevalence of infection in the wider population of yellow-eyed penguins in New Zealand.

Materials and Methods

This study involved 178 yellow-eyed penguins identified at routine nest checks on the South Island (n=133), Stewart (n=33) and Codfish Islands (n=12) during the November 2006 to January 2007 breeding season (Figure 1). Penguins on Stewart Island were monitored along the north-eastern Anglem coast while those on Codfish Island were located at Sealers Bay. South Island penguins were sampled from Oamaru, Otago Peninsula and the Catlins.

Physical examination

Within the study group, physical examinations were performed by a single veterinarian on a total of 94 yellow-eyed penguins to compare body condition and assess clinical signs, comprising birds from Stewart Island (n = 12 adults and 14 chicks) and the Catlins (n = 32 adults and 36 chicks). Body condition was determined subjectively by palpation of the keel, spine and legs, and a body condition score (BCS) given out of 9, with higher scores denoting better body condition and higher fat reserves. Injuries and abnormalities were recorded at the time of examination, and each individual was identified by flipper band or microchip.

Blood collection and smear preparation

Blood samples were collected using the ulnar or brachial vein from a total of 119 yellow-eyed penguins, from the South Island (n=95), Stewart (n=12) and Codfish Island (n=12). Blood samples were stored in lithium heparin vials and frozen at -20°C. Thin blood smears were prepared at the time of collection from a total of 49 penguins, from the Catlins (17 adults and 18 chicks) and Stewart Island (12 adults and 2 chicks). Blood smears were obtained from chicks over 10 days old. The blood smears were air dried, fixed in absolute methanol and subsequently stained with Wright's stain. Each smear was examined under a light microscope for 10 minutes, initially at 400x, then 1000x.

Post-mortem examination

Birds which died on Stewart Island (n=32) during the 2006/07 season and were in suitable condition (n=25) underwent post-mortem examination using standard methods (Rae 2006). Samples of liver, spleen, lung, kidney and brain were frozen (n=7) or placed in 10% buffered formalin (n=14) from where liquid nitrogen or formalin was available in the field.

Molecular studies

DNA was extracted from frozen heparinized whole blood or frozen tissues using a Qiagen DNeasy Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The presence of the cytochrome b gene of *Leucocytozoon* was identified using a single-tube nested PCR as described by Hellgren *et al*. (2004) without modification. Initial primers (HaemNFI [5'-CATATATTAAGAGAAITATGGAG-3'] [I = a universal base, inosine] and HaemNR3 [5'-ATAGAAAGATAAGAAATA CCATTC-3']) were used to amplify parasite mitochondrial DNA, then for the second PCR primers (HaemFL [5'-ATGGTGTTTTAGATACTTACATT-3'] and HaemR2L [5'-CATTATCTGGATGAGATAATGGIGC-3']) for *Leucocytozoon* spp. were used.

As per Hellgren *et al*. (2004) the first PCR was performed in volumes of 25ul, which included 50ng of genomic DNA, 1.25mM of each deoxynucloside triphosphate, 1.5mM $MgCl_2$ 1xPCR (Applied Biosystems Inc, Forster City, California, USA), 0.6mM of each primer, and 0.5 units *Taq* DNA polymerase. The PCR was conducted

using the following conditions: 30 sec at 94 C, 30 sec at 50 C, and 45 sec at 72 C for 20 cycles. Samples were incubated before the cyclic reaction at 94 C for 3 min and after the cyclic reaction at 72 C for 10 mins. From the first PCR reaction 1ul of PCR product was used as a template for the second PCR in 25ul volumes using the same proportions of reagents as in the initial PCR reactions. The thermal profile of the PCR was identical to the initial PCR but performed for 35 cycles instead of 20 cycles. To confirm successful amplification 1.5ul of the final PCR product was run on a 2% agarose gel prior to purification and sequencing.

All *Leucocytozoon* positive PCR amplicon samples were purified using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA) and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, Foster City, California, USA) to confirm genomic sequence.

Analysis of *Leucocytozoon* sequences (416 bases) obtained from yellow-eyed penguins was compared to those from Fiordland crested penguins and other published sequences available from Genbank. Alignments were performed using Clustal W (Higgins *et al*. 1994), and a phylogenetic tree generated using a Jukes-Cantor distance model and neighbour-joining method in Geneious™ (Biomatters, Auckland, New Zealand). Bootstrap values were generated from 100 cycles.

Statistics

The small sample size limited statistical analysis of results. Freecalc 2 (Cameron 2001), Win Episcope 2.0 (Blas 2000) and reference tables (Beyer 2005) were used to generate 95% confidence intervals for the true prevalence based on a low expected prevalence (3-5%). The range of the true prevalence is reported at the 95% confidence interval.

Results

Physical examination

All 32 monitored chicks on Stewart Island died during the 2006/07 breeding season. They were pale, grossly underweight at 100-200g with poor feather growth, and exhibited lethargy, poor body condition (BCS <2/9) and muscle atrophy. Seven chicks (22%) developed swollen eyes and peripheral oedema. Open mouth breathing and respiratory distress were often noted just prior to death. Deaths occurred at a mean of 10 days old, with a range of 2-108 days, as shown in Figure 2. Chicks died within 12-72 hours after the onset of clinical signs. Two chicks from the Catlins were observed with poor growth and dyspnoea and subsequently died, but were not available for post-mortem. Similar illness was not observed in penguins at other mainland sites. All other chicks examined in the Catlins (n=34) were bright and active, in good body condition (mean BCS 5/9, range 4-8) and complete plumage.

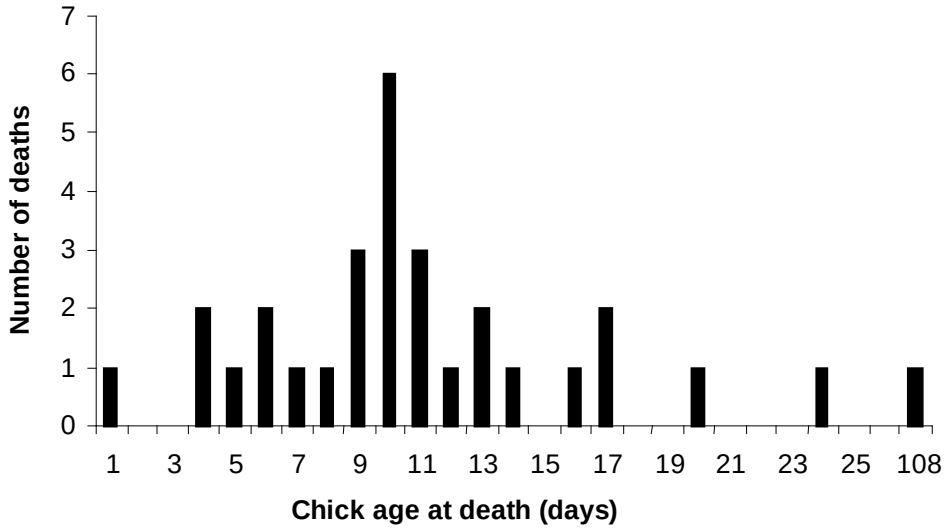

Figure 2. Distribution of age at death for chicks on Stewart Island from November 2006 to February 2007.

Physical examination of adult penguins on Stewart Island found that 11 of 12 (92%) possessed significant fat reserves and higher body condition scores (mean BCS 7/9) than adults in the Catlins (n=32), which were in moderate condition (mean BCS 5/9, range 4-7). Occasionally injuries or abnormalities were identified including trauma (n=3) and an impacted uropygial gland (n=1). Nesting adults on Stewart Island had petechial haemorrhages around the eyelids which were not observed in a non-nesting vagrant on Stewart Island or at other colonies.

Blood smears

Examination of blood smears taken from birds from the Catlins and Stewart Island found no evidence of parasitaemias, and no abnormalities of red cell morphology by light microscopy. Of 12 Stewart Island adults that were negative for *Leucocytozoon* on blood smear examination, 10 (83%) were positive for *Leucocytozoon* by PCR from

the same sample. Blood smears from 2 chicks on Stewart Island at 21 days old did not show parasitaemia, however one chick later died of severe leucocytozoonosis at 108 days of age and the second was lost to follow-up prior to fledging.

Post-mortem findings

Post-mortem findings from 25 chicks which died on Stewart Island included generalised pallor (n=22, 88%), splenomegaly (n=4, 16%) and hepatomegaly (n=5, 20%). Variable ecchymotic haemorrhages of the pericardial, serosal and subcutaneous surfaces (n=3, 12%), and pulmonary haemorrhage (n=2, 8%) were seen in severe cases. The stomach was full in 7 chicks, containing fish material and/or nesting material, empty in 6 others, and unreported in the remaining 12. The oldest chick that died at 108 days of age exhibited poor body condition, pale musculature, extensive subcutaneous, serosal and pericardial ecchymotic haemorrhages, splenomegaly and pulmonary haemorrhage (Figure 3). Histology from 2 of 14 (14%) chicks showed severe, disseminated megaloschizont formation characteristic of leucocytozoon in the liver, spleen, lung, kidney and other tissues (Figure 4). These birds were aged 22 and 108 days, and were the oldest chicks sampled. The remaining samples showed neither primary schizont or megaloschizont formation. In 11 cases, post-mortem was not followed by histology due to poor availability of formalin in the field.

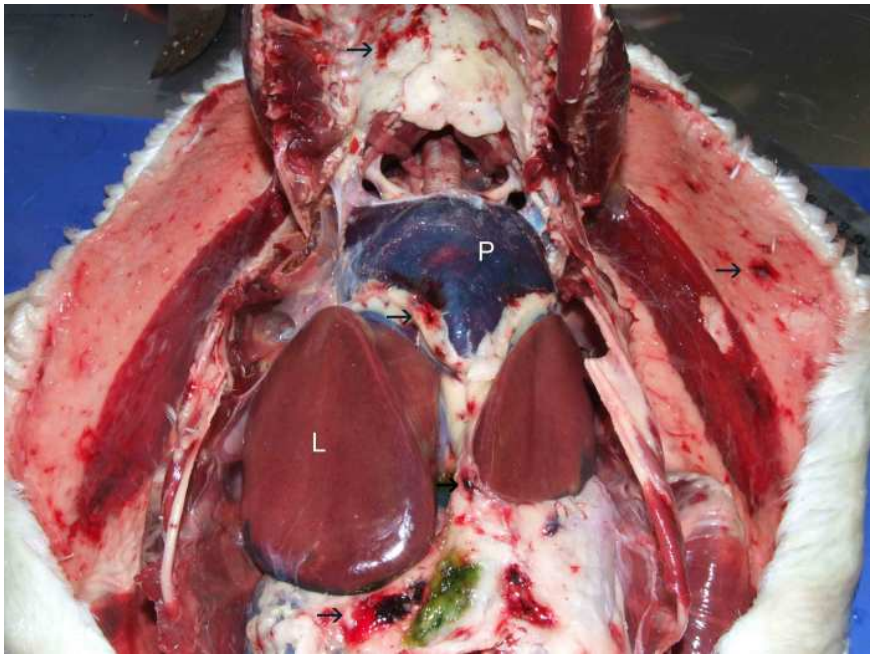

Fig 3. Gross post mortem appearance of a 108 day old chick in ventral recumbency showing disseminated petechial haemorrhage (arrows). The liver (L) was mildly enlarged and haemorrhage beneath the pericardium (P).

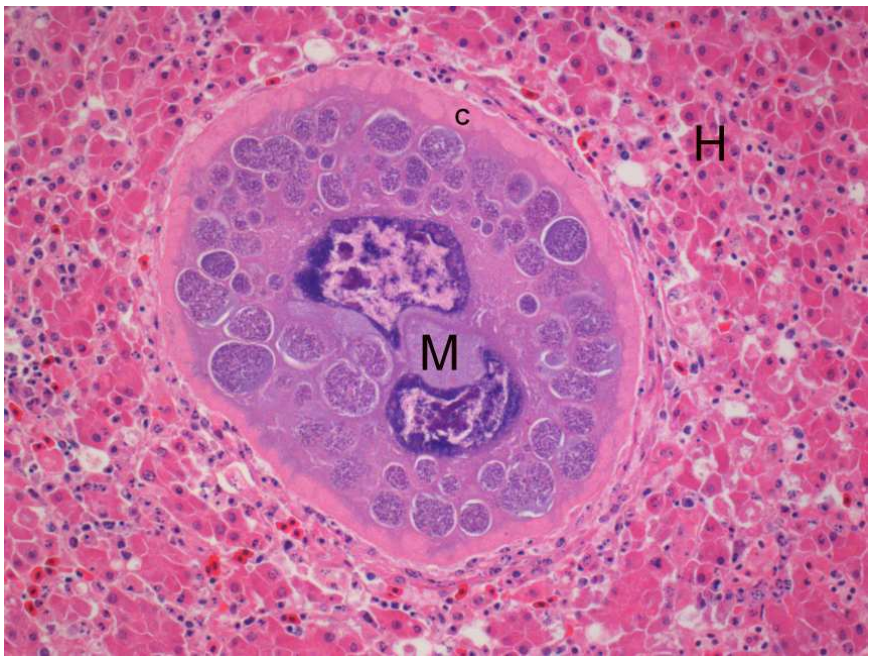

Fig. 4. Photomicrograph showing a hepatic megaloschizont (M) from the same chick, separated from hepatocytes (H) by a thick capsule (C) (200x).

Molecular studies

Leucocytozoon DNA was amplified from 10 of the 119 (8%) blood samples as shown in table 1, reflecting infection in 10 of 12 (83%) blood samples taken from adults on Stewart Island. Further, thawed tissue samples from 7 of 7 (100%) chicks in the post mortemed group on Stewart Island (n=25) were strongly positive for *Leucocytozoon* DNA, of which 1 of 5 was also confirmed histologically by the presence of megaloschizonts in tissues. *Leucocytozoon* was not detected by PCR in the blood samples obtained from 107 penguins from Codfish Island (12), Catlins (30), Otago Peninsula (33) and Oamaru (32).

Table 1. Body condition, blood smear and PCR results for *Leucocytozoon* from penguins on the South Island, Stewart and Codfish Island during the study period.

	Examined	Body condition^	Blood smear♠	Blood PCR♠	True Prevalence♦
Adults					
South Island	32	-	0/17	0/95	0-2.8%
Oamaru	0	N/a	0	0/32	
Otago Peninsula	0	N/a	0	0/33	
Catlins	32	5/9 (4-7)	0/17	0/30	
Stewart Island	12	7 /9 (5-9)	0/12	10/12	51.6-97.9%
Codfish Island	0	N/a	0	0/12	0-21.25%
Total Adult	44	-	0/29	10/119	4.5-16.2%
	Examined	Body condition^	Blood smear*	Tissue PCR♠	
Chicks					
Catlins	36	5/9 (4-7)	0/18	n/a	
Stewart Island	14	2/9 (0-5)	0/2	7/7	
Total penguins	94	-	0/49	-	

^Body condition scores: mean and range
*smears collected for penguins >10 days old
♠ results given as positive/total sampled
♦ at 95% level of confidence

Statistics based on PCR results

The test prevalence of *Leucocytozoon* in the yellow-eyed penguin population was 8.4% with the true prevalence of *Leucocytozoon* falling between 4.5-16.2 % at the 95% level of confidence. The test prevalence of *Leucocytozoon* on Stewart Island was 83.3% with the true prevalence of infection in this population falling between 51.6-97.9% at the 95% level of confidence. Although no *Leucocytozoon* was detected on Codfish Island in this survey, this result may reflect the small sample size with a true prevalence of up to 21.25%. Similarly, the presence of infection on the South Island is likely to be very low with this survey finding no infections suggesting a true prevalence range up to 2.8%.

Sequencing

Sequences of *Leucocytozoon* DNA from yellow-eyed penguins formed a distinct cluster compared to the other leucocytozoon isolates selected from Genbank (Figure 5). *Leucocytozoon* isolated from two Fiordland crested penguins, a closely neighbouring species known to breed on Stewart Island, was not directly related to those found in yellow-eyed penguins. Interestingly, the sequences of Fiordland crested penguins were not directly related to each other, instead neighbouring *Leucocytozoon* sequences obtained from a tit (species unknown) and Marsh wren (*Cistothorus palustris*) (Genbank refs DQ847244.1 and DQ847212.1).

There is currently no sequence data for *L. tawaki* so comparisons made in this paper are based on *Leucocytozoon* DNA obtained from Fiordland crested penguins. Further

investigation is required to confirm sequence data with histological lesions for *Leucocytozoon* in Fiordland crested penguins. Such information may provide greater scope for determining the relationship of *Leucocytozoon* sp. from Fiordland crested and yellow-eyed penguins.

Discussion

This study identifies an unusual geographic variation in the prevalence of infection of yellow-eyed penguins with *Leucocytozoon* that correlates with an observed pattern of low reproductive success. A definitive link between *Leucocytozoon* and nestling deaths was not confirmed although histological examination suggests that older chicks (n=2) died with disseminated leucocytozoonosis. *Leucocytozoon* infection was characterised by acute signs in chicks with megaloschizont formation in tissues and chronic infection in clinically normal adults detected by blood PCR, but not smears.

The identification of *Leucocytozoon* in 7 of 7 (100%) chicks and 10 of 12 (83%) adults on Stewart Island showed that infection was far more prevalent on this island than previously realised. Infection with *Leucocytozoon* was limited exclusively to Stewart Island, with no positive results found at any other location, including Codfish Island. Previous identification of 3 cases of *Leucocytozoon* on Codfish Island by Alley (2005) strongly suggests that our sample size (n=12) was insufficient for detection at the current prevalence, but also raises the possibility of intermittent infection occurring with dispersal of infected juveniles and seasonal changes in vector transmission.

The distribution in this study correlates with low reproductive success on Stewart Island, which has been between 20.7-33.3% since 2003, compared to the Catlins (60-99.1%) and Codfish Island (48.1-56.6%) over the same period. Interestingly, breeding success in the Bravo Islands, a small cluster of islands within the Stewart Island group, more closely resembled that on Codfish Island rather than Stewart Island (King 2007). The reasons for this distinct variation in breeding success and *Leucocytozoon* prevalence are unclear but is suggestive of localised factors on Stewart Island.

Investigation into the causes of chick mortality has been stimulated in part by evidence that predation by cats on Stewart Island has been minimal, whereas predation by cats and mustelids on the South Island, where nesting sites are often adjacent to farmland, tourism or recreational areas, is significant (King 2007). On Stewart Island, starvation and disease are proposed as key factors in failure to fledge (Darby and Seddon 1990; Massaro and Blair 2003; King 2007).

Starvation may have been a contributing factor to chick mortalities. It is possible that there was sufficient nutrition to maintain condition in adults, but not rapidly growing chicks. Starvation may be due to poor delivery, acceptance, digestion or quality of food. Evidence of recent feeding activity in chicks indicated by stomach contents at post-mortem suggests that active food delivery occurs in many unwell chicks, so that starvation may be a secondary factor. A concurrent study by Browne (*pers comm.* T. Browne*)* found that adults on Stewart Island predominantly foraged on blue cod, however the nutritional significance of this remains unknown.

The high prevalence of *Leucocytozoon* infection in the chicks from Stewart Island suggests a role for this haemoparasite in the chick deaths. However, little is known about the physiological, behavioural or ecological cost of haemoparasites in wild hosts (Atkinson 1991). In chicks, rapid growth and moulting confer a considerable degree of physiological stress. Further, excessive immune response to pathogens may directly cause inappetance, and direct nutrients away from homeostasis and production (Deitemeyer 2005). Allison *et al*. (1978) also suggested that heavy *Leucocytozoon* infection may render chicks more susceptible to secondary infection or stress. Given the choice to breed at nearby sites which feature greater reproductive success such as Codfish and Bravo Islands, adults on Stewart Island maintain a high degree of site fidelity, which may be an innate response or reinforcement by an environment which allows them to maintain condition throughout the breeding season.

Yellow-eyed penguin chicks are exposed to large numbers of biting flies from hatching and are not protected in the nest by parents as observed in Fiordland crested penguins (Allison 1978). Early inoculation combined with short prepatent periods, which have been recorded as early as 4 days in some *Leucocytozoon* spp., could account for early chick deaths due to *Leucocytozoonosis* (Desser 1967; Khan and Fallis 1970; Fallis *et al*. 1974). The suspected vector, *Austrosimulium ungulatum*, increases feeding activity with warmer temperatures, peaking in early morning and late afternoon over summer, coinciding with chick rearing (Allison *et al*. 1978). Occasional conjunctival petechial haemorrhages observed in nesting adults on Stewart Island may be caused by biting vectors. Thus, adults may be exposed to low levels of re-infection throughout the year, stimulating immunity, while chicks are exposed to

high levels of *Leucocytozoon* during the peak transmission period of the breeding season (Allison *et al*. 1978). The overwhelming presence of simuliid vectors on Stewart Island is suggestive of its role as a vector, however conclusive evidence would require transmission studies.

The presence of detectable circulating *Leucocytozoon* in chronic infections, by blood smear or PCR, indicates ongoing schizogony (Fallis *et al*. 1974). Low-level parasitaemias in clinically normal adults, below the detectable limit of blood smears but within the range of PCR, suggests subclinical infection but may also be due to the recrudescence of latent infection due to immunosuppressive factors, or the detection of inactive latent infections by the sensitivity of the test (Cranfield *et al*. 1994; Graczyk *et al*. 1995b; Brossy *et al*. 1999). The expression of subclinical infection is likely to be influenced by stressors such as reproductive activity, prey shortage, environmental instability, concurrent disease and the threat of predation.

Although we have documented leucocytozoonosis in yellow-eyed penguins, it remains unclear what role *Leucocytozoon* infection plays in reproductive success and mortality. The clinical signs exhibited by chicks are consistent but non-specific, with poor growth and development a common finding in a number of neonatal conditions. *L. simondi* and *L. smithi* both cause lethargy, inappetance, dyspnoea, anaemia and death and are associated with splenomegaly and hepatomegaly as seen in yellow-eyed penguins (Fallis *et al*. 1974). Infection with *L. smithi* in turkeys (*Meleagris gallopavo*) produced respiratory distress caused by alveolar capillary blockage, but this was not observed histologically in penguins (Siccardi *et al*. 1974).

The *Leucocytozoon* species found in this study exhibited a large megaloschizont tissue phase, which may be a factor in its association with morbidity and mortality. Fallis *et al*. (1974) noted that pathogenic species, including *L. simondi*, *L. caulleryi* and *L. sakharoffi*, feature megaloschizont formation while those species that cause less severe disease do not. The observed anaemia in acute infections is thought to be caused by anti-erythrocytic factors released at the time of schizont rupture, although confirmation of this was outside the scope of this study (Atkinson and Van Riper III 1991). Further investigation is warranted to quantify and characterise anaemia to determine its role in penguin mortalities.

The presence of highly similar *Leucocytozoon* sequences in adults and chicks indicates that adults may serve as a reservoir host for the infection of chicks. The penguin host maintains infection during the non-breeding season, so the introduction of dispersing, infected penguins in a naïve colony with appropriate vectors could have severe and widespread effects. Comparison of *Leucocytozoon* isolates between yellow-eyed penguins and two Fiordland crested penguins found dead near the Foveaux Strait showed distinct divergence at the molecular level, suggesting infection with a separate species to that found in yellow-eyed penguins. Morphological identification was not available for infections in either species given the absence or low level of circulating gametocytes. There have been no studies documenting the prevalence or clinical effect of *L. tawaki* in Fiordland crested penguin for comparison, despite its endangered status.

Leucocytozoon is considered host specific at the family level, and Allison *et al*. (1978) demonstrated vector transfer of *L. tawaki* from a Fiordland crested to a

juvenile little penguin (*Eudyptula minor*), so it is possible that the close nesting proximity of these species and high vector density on Stewart Island could allow interspecies spread. Despite this, our results indicate that there may be two separate, endemic *Leucocytozoon* infections within the yellow-eyed and Fiordland crested penguins in this region.

The impact of ongoing chick losses on the survival of the yellow-eyed penguin population is ameliorated by the longevity of adults, but continued chick losses of this magnitude will result in a high extinction probability for these populations. The Stewart Island and Codfish Island breeding group represent a significant population for the species and dispersing juveniles may travel to both the South Island and the subantarctic islands (Anon. 1991). Stewart Island yellow-eyed penguins may also represent a genetically distinct group which is highly important in an endangered species with a limited breeding pool (Triggs and Darby 1989).

We found that PCR was superior to peripheral blood smears in the detection of *Leucocytozoon* from clinically normal birds. This finding is consistent with other studies surveying low-level parasitaemias (Jarvi *et al*. 2002; Richard *et al*. 2002; Swinnerton *et al*. 2005). Jarvi *et al*. (2002) found that the sensitivity (Bayes' theorem) of detecting the chronic phase of *Plasmodium* by microscopy was 0.27 (0-0.67) compared to 0.61 for PCR (0.2-0.87). Hellgren *et al*. (2004) showed 100% positive amplification from circulating *Leucocytozoon* levels over 1 gametocyte per 10 000 blood cells, but only 67% with levels of 1 per 100 000 blood cells for the primers used in this study.

One chick which tested negative on PCR and blood smear at 21 days, died of overwhelming leucocytozoonosis at 108 days old on the day it was due to fledge. The negative result may have been due to insensitivity of the test, lack of circulating gametocytes in an early infection or infection that occurred after the time of sampling. Allison *et al*. (1978) did not detect gametocytes in 9 chicks less than 3 weeks old despite findings in older chicks, suggesting their absence may be a factor of age and stage of infection. The short window when life stages of *Leucocytozoon* are present in peripheral blood made detection by smears difficult and may also cause underestimation of prevalence determined by nested PCR of blood samples (Jarvi *et al*. 2002; Hellgren *et al*. 2004; Swinnerton *et al*. 2005).

This study has provided a single prevalence of *Leucocytozoon* on Stewart Island and its surrounding colonies, however wider studies are needed of Stewart Island, the subantarctic islands, which constitute the bulk of the yellow-eyed penguin population, and of other seabirds on these islands which may represent alternate hosts. It may also be useful to determine the incidence of *Leucocytozoon* infection over multiple years to reliably estimate its effect on the population dynamics of the yellow-eyed penguin.

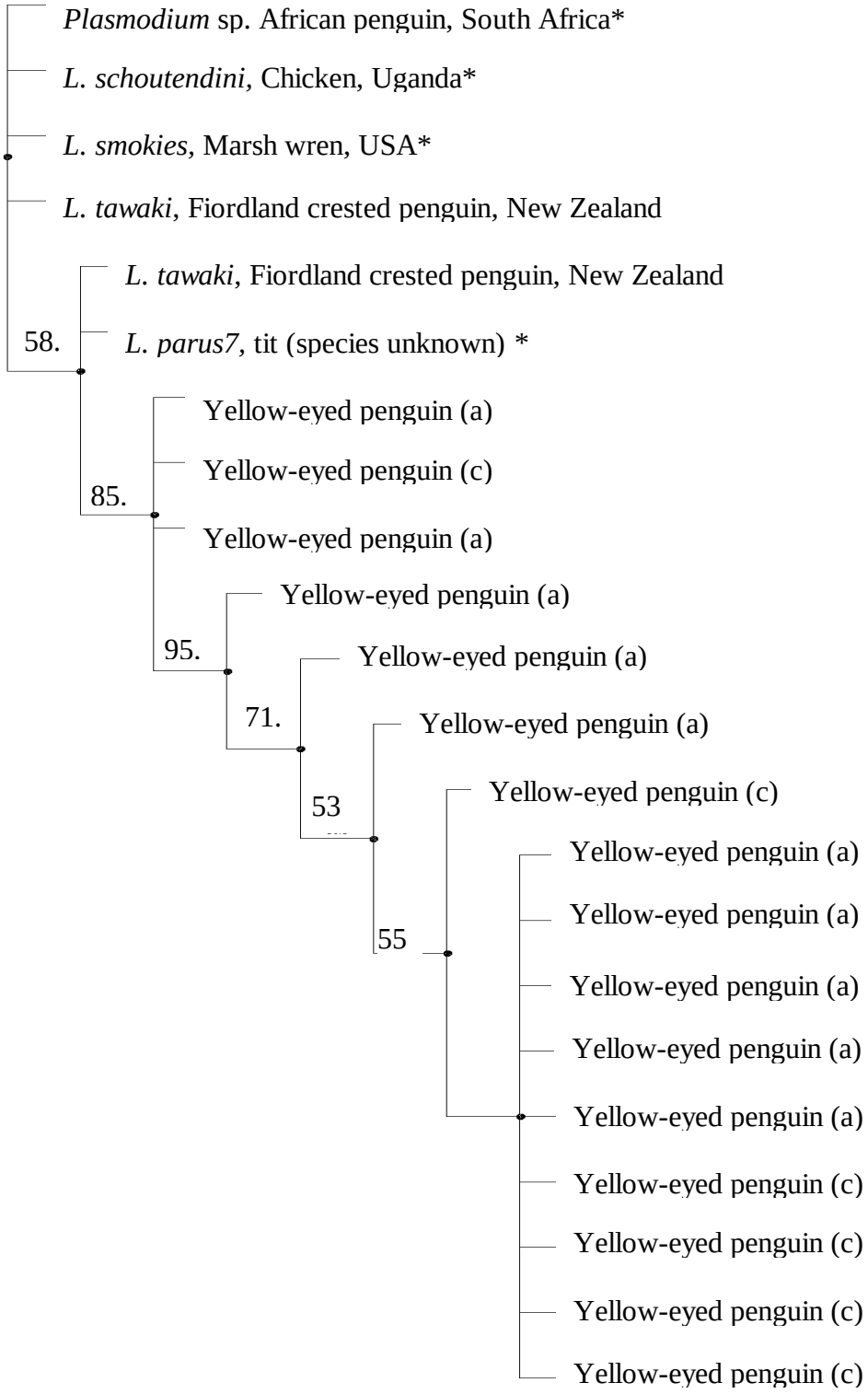

Figure 5. A neighbour-joining tree from cytochrome B gene DNA sequences of leucocytozoon spp. from wild caught penguins (n=18) and previously reported sequences from Genbank (n=4, denoted by *). Bootstrap values from 100 cycles are shown at the branches. Nodes are denoted by •. (a) Adult (c) chick.

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Chapter 3

The histopathology and fine structure of a novel *Leucocytozoon* sp. from the endangered yellow-eyed penguin (*Megadyptes antipodes)*: megaloschizonts and gametocytes.

For submission to *Avian Pathology*

Abstract

The morphological characteristics of *Leucocytozoon* isolates associated with chick mortality in yellow-eyed penguins more closely resembled *L. simondi* and *L. sakharoffi* than its geographic neighbour *L. tawaki*. Megaloschizont formation occurred mainly in hepatic and splenic tissue, and was observed in most other tissues in heavy infection, but never in the brain. The megaloschizonts grew to 440µm and were observed releasing occasional intact cytomeres. Round gametocytes were observed within leucocytes in 1 of 8 cases studied. Further investigation is required to determine the life-cycle and impact of this novel *Leucocytozoon* in this endangered penguin species.

Introduction

Leucocytozoon was first described in yellow-eyed penguins in 2005 when it was seen in the tissues of 8 dead chicks from Stewart and Codfish Islands (Alley 2005). Previous descriptions of *Leucocytozoon* have been made in Fiordland crested

(*Eudyptes pachyrhyncus*) (Allison *et al.* 1978), Macaroni (*Eudyptes chrysolophus*) (Peirce *et al.* 2005) and African penguins (*Spheniscus demersus*) (Earle *et al.* 1992) but in these earlier cases the organisms were considered incidental infections.

In the affected yellow-eyed penguins the organism was associated with regional, acute chick mortality featuring anaemia and generalised subcutaneous ecchymotic haemorrhage. Since the affected birds nest on Stewart Island in close proximity to Fiordland crested penguins, in the presence of a significant simuliid population, it was thought that cross-infection between these species of penguin was a possibility. This study aimed to describe the histology and fine structure of the *Leucocytozoon* sp. found in yellow-eyed penguins and make comparisons with prior descriptions of *L. tawaki* and other *Leucocytozoon* spp.

Methods

Histological sections demonstrating multiple intact *Leucocytozoon* megaloschizonts from 8 wild yellow-eyed penguins with leucocytozoonosis from Stewart and Codfish Island were reviewed from a collection representing all 45 yellow-eyed penguin post-mortem submissions from this region during the 2005-2008 period. The collection contained 12 cases with histological evidence of *Leucocytozoon* and selection was based on availability and the presence of multiple megaloschizonts. All available organs were examined including liver, spleen, lung, heart, brain and intestine. Tissues fixed in 10% buffered formalin were prepared routinely for histopathology and paraffin embedded sections were cut at 4 µm and stained with haematoxylin and

eosin. Tissue sections were screened for the presence of schizonts and megaloschizonts at 40x magnification by light microscopy.

Photomicrographs of 17 hepatic, splenic and renal megaloschizonts from 3 separate cases were obtained to allow measurement of morphological features. Each megaloschizont was measured at 1000x using Olympus AnalySIS five software[†].

Material for electron microscopy was selected from spleen and liver tissue, fixed in 10% buffered formalin for over 48 hours, then transferred to 3% glutaraldehyde in Karnovsky fixative for 48 hours. Specimens were rinsed in pH 7.2 0.1M phosphate buffer and stained with 1% osmium tetroxide in double distilled water for 1hr. After triple rinsing with the above buffer, specimens were put through a dehydration series of 10mins each in 25%, 50%, 70%, 90% and 100% acetone, followed by 1 hr in dry acetone. They were then embedded in Procure 812 resin. Sections were cut with an ultramicrotome, and stained with uranyl acetate and lead citrate before examination under a Philips 200 transmission electron microscope.

Results

Leucocytozoon spp. megaloschizonts from 8 cases were present in the liver (n=7), spleen (n=7), kidney (n=5), lung (n=3), heart (n=2) and intestine (n=2) (Table 1). In severe cases, disseminated megaloschizont formation was found in all visceral tissues except the brain. Primary schizonts were not identified in any tissues despite the presence of numerous megaloschizonts and gametocytes within tissues.

[†] Olympus Imaging Corporation, Shinjuku-ku, Japan.

The megaloschizonts in all tissues examined consisted of round to ovoid, intracellular, protozoal cysts with a well defined capsule, markedly hypertrophied nucleus and multiple cytomeres causing severe distortion of the host cell (Figure 1). They had a 3-24µm capsule consisting of concentric, loosely arranged eosinophilic fibres containing occasional haemosiderin granules. In many cases, capsular material could be divided into an outer, loosely arranged eosinophilic border, and a more dense, eosinophilic to basophilic internal capsule which extended internally to create divisions between cytomeres and became homogenous with the host cytoplasm. In early megaloschizonts with thick capsules, invaginations of the inner basophilic layer into the outer capsule were also present.

Developing cytomeres ranged from 3-99µm in diameter and were very well demarcated, with a thin, variably stained border containing numerous basophilic 3-4µm round to rod-shaped granules. The number and size of cytomeres varied widely with section and schizont maturity. One mature megaloschizont contained in excess of 70 cytomeres but most mature megaloschizonts contained between 16 and 38 cytomeres, with fewer cytomeres reflecting a larger cytomere diameter.

In well-preserved, early to mature schizonts, the 'hypertrophied host nucleus' (Desser 1970) or 'central body' (Huff 1942) contained one or more sparse clusters of basophilic material, separated from cytomeres by a wide border of dense homogenous eosinophilic cytoplasm. As schizonts matured, much of this material became compressed or was lost between growing cytomeres. Within the host nucleus of some well-preserved, early megaloschizonts there were singular 5-7µm vacuolar structures containing diffuse lightly basophilic material and a central eosinophilic granule,

reminiscent of a typical gametocyte nucleus. Where multiple nuclear regions were present a similar structure could be seen in each. Once fragmentation or disruption of the nuclear region occurred, or perhaps with variation in cut sections, this structure was lost.

Host reaction to megaloschizont formation was not observed in any tissue, except where rupturing schizonts were present. Recently ruptured schizonts evoked a moderate inflammatory reaction characterised by occasional heterophils, lymphocytes and macrophages. Increased extramedullary haematopoiesis was observed in 5 of 9 cases (56%) in the liver and spleen. Excessive haemosiderin deposition was also noted in the 2 most severe cases.

Table 1. Summary of tissue distribution of megaloschizonts in 8 yellow-eyed penguins submitted for post-mortem.

	Liver	Spleen	Kidney	Lung	Heart	Brain
Case 1	++++	++++	++++	++++	++	-
Case 2	++++	+++	-	+++	-	-
Case 3	+	++++	++++	+	-	-
Case 4	+	++++	++	-	-	-
Case 5	-	++++	+	-	-	-
Case 6	+++	++	-	-	++	-
Case 7	++	+	++	-	-	-
Case 8	+	-	-	-	-	-

- = no megaloschizonts + = 1-2 megaloschizonts / 20x field

+++ 5-10 megaloschizonts /20x field ++ 3-5 megaloschizonts /20x field

++++ greater than 10 megaloschizonts /20x field.

Megaloschizont diameter varied with maturity, tissue of origin and section. Diameter ranged from 194µm to 440µm in 17 megaloschizonts measured from 3 separate cases with a wide degree of variation within and between cases. Hepatic megaloschizonts were up to 440µm and generally circular to ovoid, while renal megaloschizonts were

distorted into elongated structures around tubules and glomeruli. Similar distortion was seen in megaloschizonts in the heart and intestinal wall.

The size of the nuclear region was also highly variable, ranging from 68µm to 189µm (26% and 43% of megaloschizont diameter respectively). There were no consistent features associated with the formation of numerous small cytomeres compared to fewer large cytomeres, or with fragmentation of the nuclear region. A summary of basic morphometrics is shown in table 2.

Table 2. Morphometrics based on 17 megaloschizonts from 3 penguins.

	Mean +/- SD*	Range
Megaloschizont diameter	356 +/- 47um	264-440um
Capsule thickness	14 +/- 4um	8-24um
Host nucleus diameter	138 +/- 31.3um	68-189um
Cytomere diameter	36 +/- 7.8um	3-99um
Cytomeres per megaloschizont	27 +/- 6 cytomeres	16-38

*standard deviation

Megaloschizonts appeared to be contained within hepatocytes and endothelial cells in liver tissue and within endothelial cells in other tissues, however the extent of cellular distension and hypertrophy made identification of the host cell difficult.

The varied appearance and changing structure of the megaloschizonts was interpreted as a growing maturity of the organism. Four stages of growth were recognised:-

1. Early schizonts featured few, small cytomeres and contained an extensive eosinophilic perinuclear region. The host nucleus was often centralised and intact.

Cytoplasm between cytomeres was loosely arranged and merozoites were numerous but diffusely packed (Figure 1).

2. Growing megaloschizonts contained larger or more numerous cytomeres with more dense populations of merozoites. The cytoplasm between cytomeres was compressed, and the nuclear region became fragmented or distorted by an invasion of cytomeres (Figure 2).

3. Mature schizonts were packed with cytomeres containing very dense populations of merozoites, with compression of all internal host structures. Some megaloschizonts showed deterioration of cytomere walls, suggesting internal rupture preceding capsular rupture.

4. Once megaloschizonts ruptured, invasion of macrophages and occasional heterophils and lymphocytes occurred within a pocket of eosinophilic necrotic debris that resolved to form a circular to ovoid scar. Rupturing megaloschizonts appeared to release occasional intact cytomeres, although it is unclear if this was a consistent feature of this *Leucocytozoon* spp. (Figure 3).

Interestingly, of the features used in this classification, size was a poor indicator of maturity, especially in tissues such as the kidney and lung, where megaloschizont borders were often distorted by the surrounding parenchyma.

Fine Structure

The fine structure of megaloschizonts closely resembled that of *L. simondi* described by Desser (1970). The capsule consisted of a large outer fibrous layer with

invaginations of an inner filamentous layer toward the host cytoplasm (Figure 2), and provoked no visible host reaction while intact.

Mature cytomeres were contained in a pentalaminate envelope of thin outer membranes and a thicker inner layer. Cytomeres contained numerous (100+) merozoites enveloped in single plasma membranes, and numerous round and cleft-shaped vacuoles. A predominance of female merozoites with electron dense cytoplasm and a nucleus of bunched chromatin were observed. Occasional male merozoites with less dense cytoplasm and a lighter nucleus were observed within female dominated cytomeres.

The structure and hypertrophy of the host nucleus was similar to that seen by Desser (1970) in *L. simondi*, with multiple loose aggregations of chromatin and in some cases fragmentation of chromatin accumulations by compression of cytomeres. The host nucleus was bounded by a thick electron dense plasma membrane bordering a membrane rich, filamentous accumulation of cytoplasm containing dense populations of mitochondria, endoplasmic reticulum and ribosomes.

Gametocytes

Round gametocytes similar to *L.tawaki* were observed histologically in parenchymal vessels from a single yellow-eyed penguin (Figure 4). Large numbers of gametocytes were present in hepatic, renal and splenic capillaries in association with multiple megaloschizonts in the same tissues. Mature gametocytes were 16 diameter with granular basophilic cytoplasm and a 7-8um diameter central round nucleus.

Distortion of the host cell with peripheral displacement and compression of the host nucleus made cell identification difficult, however most of those observed were likely to be mononuclear leucocytes.

Gametocytes examined by electron microscopy were bounded from the host cell by a 3 layer plasma membrane (Figure 5). The parasite cytoplasm contained multiple mitochondria and plentiful ribosomes, and the parasite nucleus was bounded by an incomplete double membrane. Each nucleus consisted of multiple aggregations of chromatin within a field of electron-dense material, and a vacuolar area with sparse contents.

Discussion

The megaloschizonts of *leucocytozoon* found in yellow-eyed penguins were similar in microscopic morphology and ultrastructure to *L. simondi* and contrasted with previous findings in its geographic neighbour *L. tawaki* (Desser 1970; Allison *et al.* 1978). Our description fits the criteria of Fallis *et al.* (1974) for naming megaloschizonts, being over 100µm diameter, causing marked hypertrophy of the host nucleus and cytoplasm, and developing in reticulo-endothelial cells.

In contrast to *L. simondi*, where megaloschizonts were scarce in the liver (Fallis *et al.* 1974), the preferred sites in yellow-eyed penguins were the liver and spleen. *Leucocytozoon tawaki* in Fiordland crested penguins favoured hepatocytes and renal tubule cells as sites of primary schizogony, but megaloschizonts were not described.

Compared to *L. simondi*, megaloschizonts of yellow-eyed penguins were larger (440µm) and produced fewer cytomeres with more numerous merozoites in each (Fallis *et al.* 1974).

The absence of host reaction to both intact megaloschizonts and gametocytes is consistent with the intracellular nature of the parasite until it ruptures. Newberne (1957), but not Karstad (1965), identified mononuclear reactions only in the brain of ducks, however even in severe cases megaloschizont formation within the brain of penguins was not observed. A moderate inflammatory reaction was observed during rupture of the megaloschizonts, but was likely to be insufficient to produce the systemic clinical signs observed.

The primary mechanisms of pathology in leucocytozoonosis are believed to be a release of anti-erythrocytic factors and focal necrosis caused by schizont rupture (Cowan 1957; Kocan 1968; Khan and Fallis 1970; Maley and Desser 1977; Greiner and Ritchie 1994). Despite this, excessive haemosiderin deposition from haemolysis was only apparent in the most severe cases, and it is difficult to directly link sporadic focal hepatic necrosis and death. Generalised ecchymotic haemorrhage and peripheral oedema were seen in multiple cases and are consistent with disseminated intravascular coagulation and loss of endothelial integrity, suggesting there may be widespread endothelial damage due to toxic or merozoite-induced effects. This study did not identify histological evidence of vasculitis or endothelial damage even in severe cases of leucocytozoonosis, so further investigation of coagulopathy, tissue inflammation and toxic effects is warranted.

The increased extramedullary haematopoiesis seen in 5 of 9 cases is suggestive of a response to anaemia and excessive haemosiderin deposition, and may reflect haemolysis consistent with the release of anti-erythrocytic factors, extravascular phagocytosis of parasitised erythrocytes, or both. The limited presence of *Leucocytozoon* in the peripheral blood suggested by blood smears makes localised haemolysis or phagocytosis in the liver or spleen more likely than widespread intravascular destruction, although erythrophagocytosis was not a prominent feature in splenic sections. Bone marrow was not examined in this study but may provide useful information explaining the role of intramedullary haematopoiesis in anaemia in normal and infected penguins.

We observed distinct extensions of the inner capsule internally toward the host cytoplasm consistent with Desser's (1970) description of *L. simondi*, but there were also outward extensions through the fibrous capsule in early megaloschizonts. These invaginations are thought to represent advancement of the host plasma membrane to access nutrient exchange for the rapidly growing cell. It is not clear if this process is initiated by the parasite or the host cell seeking transport of metabolites, or if different mechanisms are used in early and mature megaloschizonts.

The classification of megaloschizont maturation used here is similar to that of Huff (1942), with the proportionately larger host nucleus persisting almost up to the point of rupture. The stimulus for the spontaneous breakdown of cytomere walls is currently unknown, but may be due to internal pressure, wall degeneration or death of the host cell. The presence of intact cytomeres in rupturing megaloschizonts requires further investigation. Cytomere breakdown did occur prior to rupture (Figure 3) but it

was not complete and was dissimilar to that described by Desser (1970). This phenomenon might suggest that, in the presence of overwhelming megaloschizont formation and an absence of hepatic schizonts, phagocytosis of intact cytomeres by macrophages may be sufficient to produce a second generation of megaloschizonts. This would be similar to the formation of primary megaloschizonts by intact cytomeres, or 'syncytia' released from hepatic schizonts (Desser 1970). Confirmation of this secondary cycle would require identification of phagocytised cytomeres and early megaloschizont development which was not observed.

Wingstrand (1947; 1948) demonstrated phagocytosis of intact syncytia in *L. sakharoffi,* which is thought to produce megaloschizonts. Without direct progression from sporozoites to megaloschizont, hepatic schizonts are likely to be present in yellow-eyed penguins but were not revealed in the current study despite repeated sections. It is clear that primary schizogony, being the initiation of infection, is not a prominent feature of the pathology created by acute, fulminating infection.

Megaloschizonts and exclusively round gametocytes have also been described in *L. sakharoffi, L. marchouxi,* and *L. podargii* (Fallis *et al.* 1974; Peirce *et al.* 1997; Adlard *et al.* 2002). *L. sakharoffi* features large megaloschizonts up to 480µm diameter without hepatic schizonts, and *L. marchouxi* produces megaloschizonts up to 210µm diameter which are most numerous in the spleen (Wingstrand 1948; Fallis *et al.* 1974; Peirce *et al.* 1997). The host effects of *L. podargii,* found in tawny frogmouth (*Podargus stigoides*), are unknown, however *L. marchouxi* has been directly associated with mortality and decreased survival of juveniles in the

endangered pink pigeon (*Columba mayeri*) (Peirce *et al.* 1997; Adlard *et al.* 2002; Peirce *et al.* 2005; Bunbury *et al.* 2007).

Fallis *et al.* (1974) postulated that round gametocytes were released from hepatic schizonts and elongate forms from megaloschizonts. Elongate gametocytes were not observed, and the presence of round gametocytes with an overwhelming number of megaloschizonts suggests they may be of megaloschizont origin, or that the majority of primary schizonts had ruptured prior to examination.

On the basis of convention and the presence of round gametocytes similar to *L. tawaki*, the *Leucocytozoon* sp. found in this study would also be termed *L. tawaki*. However, the histological and ultrastructural findings presented here indicates that it more closely resembles *L. simondi*, *L. marchouxi* or *L. sakharoffi* (Huff 1942; Wingstrand 1948; Desser 1970; Fallis *et al.* 1974; Peirce *et al.* 1997). Similarly, the clinical features of anaemia and mortality associated with infection parallel these *Leucocytozoon* spp. in which megaloschizont formation is a prominent feature. This supports the suggestion of Fallis *et al.* (1974) that the tissue phase and pathology may be linked. A concurrent molecular study has found that *Leucocytozoon* sp. DNA found in Fiordland crested and yellow-eyed penguins in southern New Zealand are dissimilar (unpublished data). Thus, further investigation is warranted to establish the life-cycles, taxonomy and pathology of *Leucocytozoon* in these penguin species.

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Figures

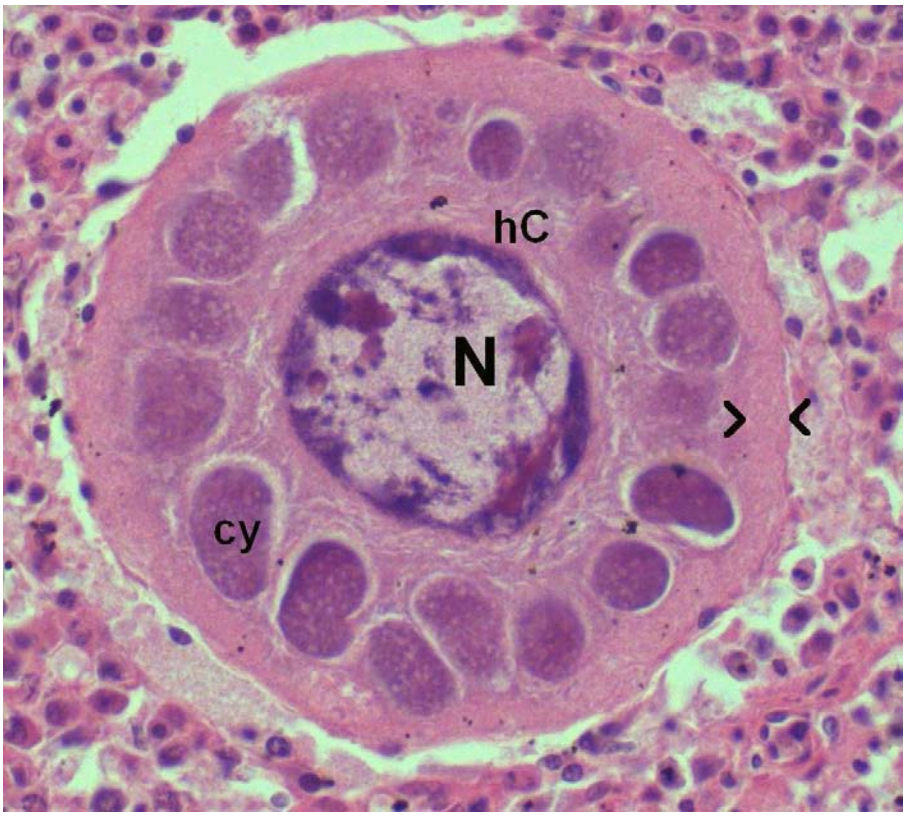

Fig 1. An early *Leucocytozoon* sp. megaloschizont in the liver of a yellow-eyed penguin. Note the thick capsule (arrowheads) and peripherally arranged cytomeres (cy). The hypertrophied host nucleus (N) contains loosely arranged chromatin and is surrounded by hypertrophied cytoplasm (hC). **300x)**

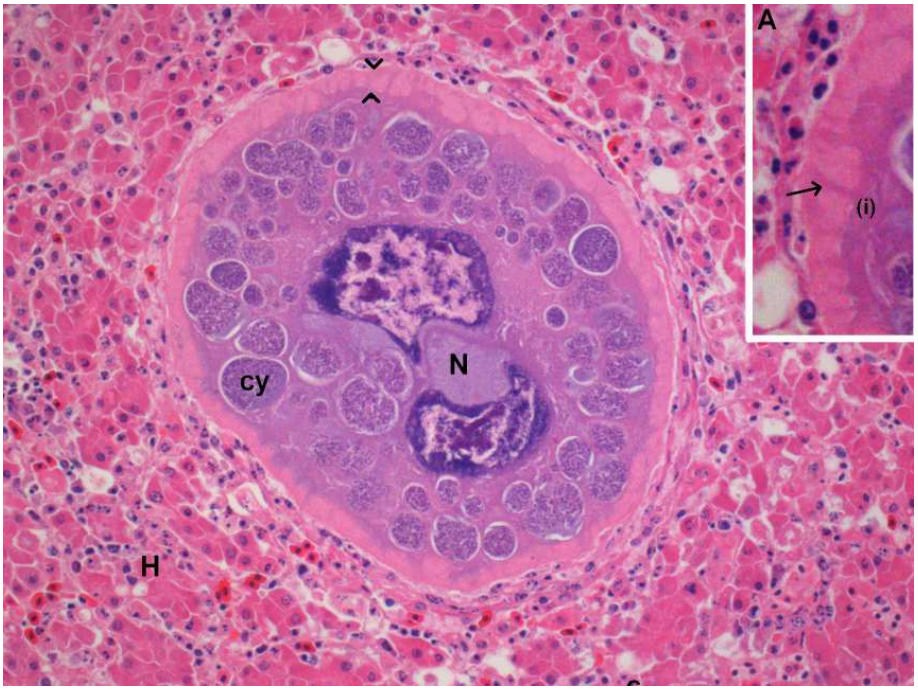

Fig2. Growing hepatic megaloschizont causing compression of surrounding hepatocytes (H) with multiple cytomeres (cy) and a distorted, fragmented nucleus (N). **(200x)** *Inset*: The capsule contains extensions (arrow) from the inner capsule/host cytoplasm (i). **(2500x)**

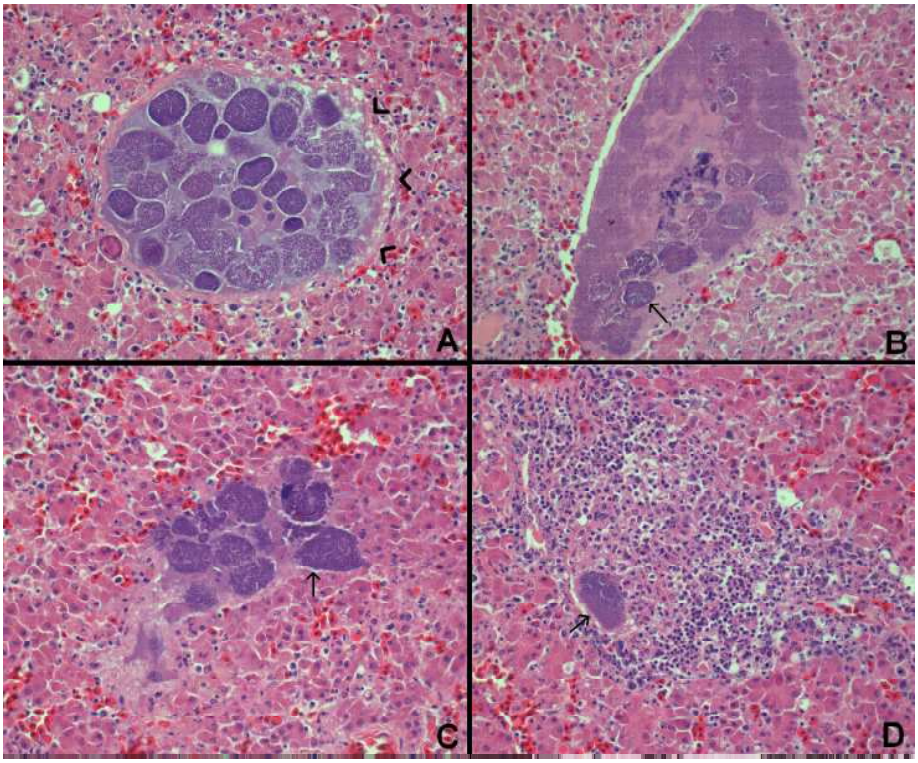

Fig 3. Degeneration of megaloschizonts beginning with (A) capsular degeneration (arrowheads) and loss of host nucleus amid compressing cytomeres with early degeneration of some cytomere walls; (B) internal cytomere breakdown prior to rupture; (C) and (D) dispersal of merozoites into surrounding sinusoids with persistence of intact cytomeres (arrow). **(150x)**

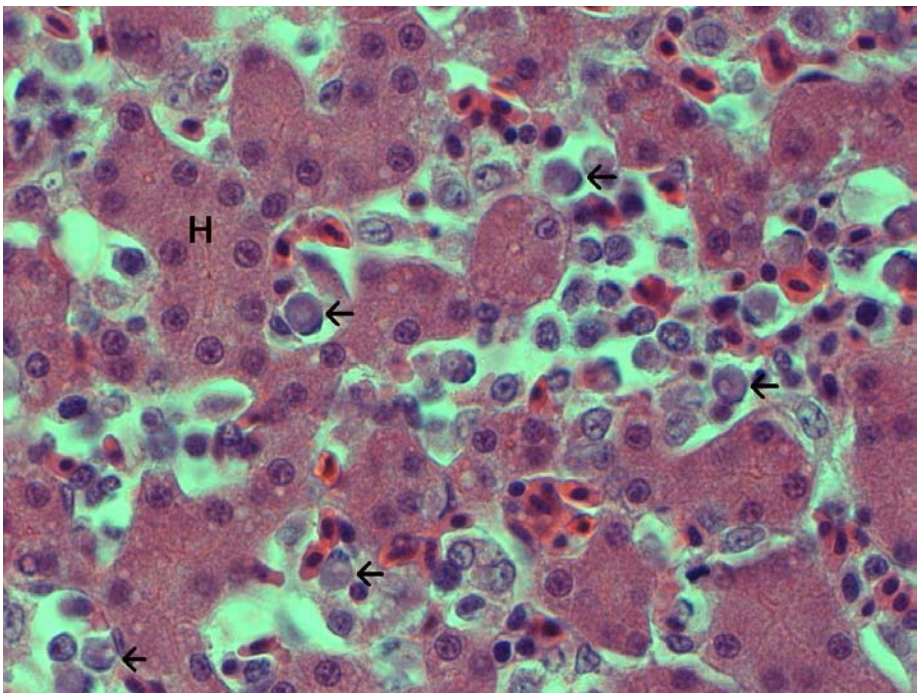

Fig 4. Round *Leucocytozoon* gametocytes (arrows) with peripherally displaced host nuclei present within hepatic sinusoids. Histological section showing hepatocytes (H) **(400x)**

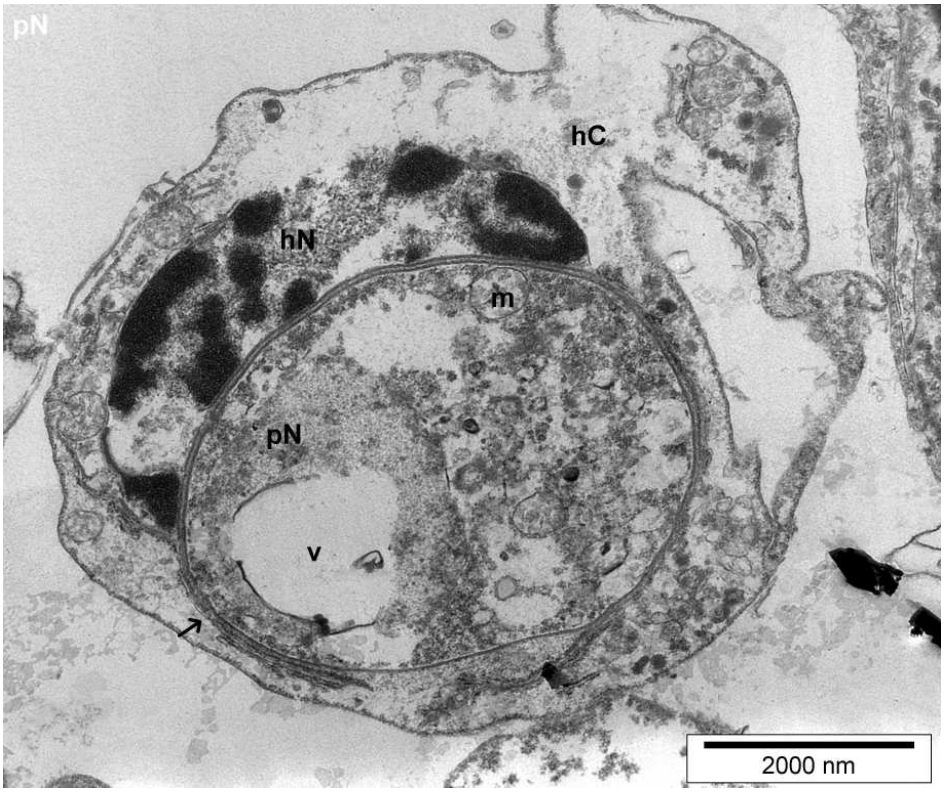

Fig 5. A microgametocyte within the cytoplasm (hC) of a mononuclear leucocyte causing displacement of the host nucleus (hN). Note the pseudopodia extending from the host cell. The parasite cytoplasm is filled with diffuse electron-dense material and mitochondria (M) while the nucleus (pN) contains a vacuolar region (v). The gametocyte is contained within a 3 layer envelope (arrow) consisting of both host and parasited derived plasma membranes. (electron micrograph)

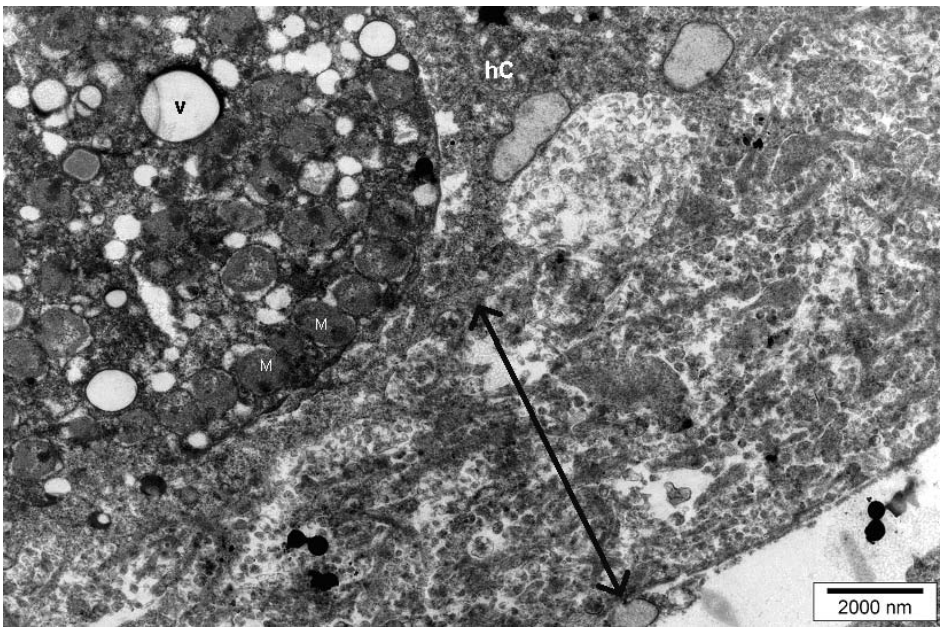

Fig 6. Megaloschizont capsule (dark arrow) bordering the host cytoplasm (hC) and a large cytomere containing predominantly female merozoites (M) and vacuoles (v). (electron micrograph).

Chapter 4

Leucocytozoonosis in a Fiordland crested penguin

(*Eudyptes pachyrhynchus*)

For submission to the *New Zealand Veterinary Journal*

Abstract

A lethargic, anaemic and underweight Fiordland crested penguin was presented to a Veterinary hospital in Auckland in 2007. Examination of blood smears revealed large numbers of *Leucocytozoon* sp. parasites and occasional, suspected *Babesia* sp. or *Plasmodium* sp. inclusions. Treatment with 30mg/kg trimethroprim and sulpamethoxazole in combination with supportive care for three weeks resulted in resolution of clinical signs and suppression of parasitaemia as assessed by blood smears. Despite the apparent success of the treatment, the penguin subsequently died. Post-mortem examination revealed a multifocal cholangiohepatitis, interstitial pneumonia and *Leucocytozoon* sp. gametocytes within blood cells in many tissues, but did not provide a definitive cause of death.

Case report

A subadult Fiordland crested penguin (Fig. 1) was presented to an Auckland Veterinary Hospital in November 2007 with a lethargic demeanour, pale mucous membranes and poor condition. The penguin had been captured on Muriwai Beach

and held for one week at a local rehabilitation centre where it was supported by gavage feeding but remained depressed and failed to gain weight.

Fig. 1 - Fiordland Crested Penguins *photographer: K. Turner*

Blood was taken from the ulnar vein for haematology and biochemistry. Haematology revealed an anaemia (PCV 17%), and blood smear examination demonstrated large numbers of round *Leucocytozoon* sp. gametocytes within blood cells, and small numbers of intracellular erythrocytic inclusions which were thought to be *Babesia* sp. *or Plasmodium* sp. (Fig. 2). Serum biochemistry including Uric acid, aspartate aminotransferase (AST) and creatinine phosphokinase (CPK) was unremarkable (Table 1).

A diagnosis of leucocytozoonosis was made and treatment initiated using 30mg/kg trimethoprim/sulpamethoxazole (Trisul Paediatric Suspension[1]) administered orally twice daily. Later, due to practical issues with dosing, the medication was changed to

[1] Pacific Pharmaceuticals, Auckland, New Zealand

trimethoprim/sulfadiazine (Tribactral 80 tablets[2]) at the same dose rate. The total duration of treatment was 4 weeks.

After ten days of treatment, the penguin's appetite and demeanour had improved. A repeat blood sample was collected and submitted to the same laboratory (Table 1). Blood smear examination showed that large numbers of round *Leucocytozoon* sp. gametocytes were present but appeared to be degenerating.

Blood smear examination after 4 weeks of treatment did not identify circulating *Leucocytozoon* sp. in peripheral blood. PCV had improved to 31% and biochemistry was not assessed. The penguin demonstrated a bright demeanour, very good appetite and good body condition. Analysis by cytochrome b PCR as described by Hellgren *et al.* (2004) of peripheral whole blood after 4 weeks of treatment was positive for *Leucocytozoon* sp. DNA. On the basis of clinical improvement, treatment was stopped and the penguin received ongoing supportive care at the rehabilitation centre.

Approximately 4 weeks after treatment was stopped, the penguin, which remained at the rehabilitation centre, died suddenly following a brief period of acute depression. A routine post-mortem was performed but did not reveal any gross abnormalities. Histopathology revealed a multifocal cholangiohepatitis characterised by heterophils and macrophages; severe, localised interstitial pneumonia and pulmonary collapse; and localised renal coccidiosis with moderate to severe tubular degeneration. *Leucocytozoon* sp. schizonts or megaloschizonts were not seen. Occasional round *Leucocytozoon* sp. gametocytes were noted within visceral vessels. It was not clear

[2] Jurox, Rutherford, NSW, Australia

whether any, or all of these lesions contributed to the death.

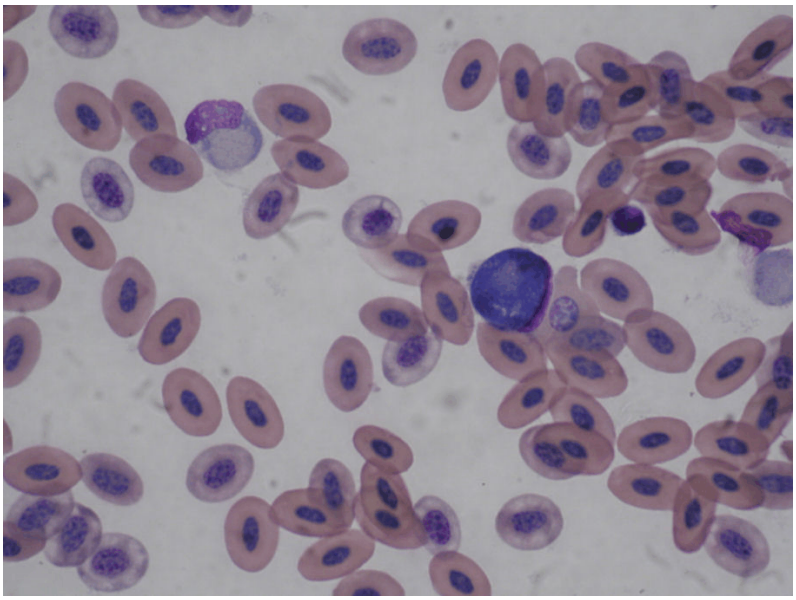

Fig. 2 - Blood smear from a Fiordland crested penguin showing an enlarged leucocyte with basophilic contents and a peripherally displaced nucleus characteristic of a round *Leucocytozoon* sp. gametocyte.

Discussion

Fiordland crested penguins are a vulnerable, endemic species to New Zealand, nesting on the South-west coast of the South Island and Stewart Island. Vagrants have been found as distant as Western Australia, 4000 km away. Population estimates range up to 3000 pairs but little is known about population trends. They are the endemic hosts of *L. tawaki* within their natural range (Fallis *et al.* 1976).

Leucocytozoon sp. is an obligate intracellular, protozoal haemoparasite of birds that replicates within avian endothelial cells and hepatocytes. Gametocytes are found in red blood cells and leucocytes. Species tend to be host specific (Bennett *et al.* 1994), and have been reported in Fiordland and yellow-eyed penguins (*Megadyptes*

antipodes) in New Zealand (Fallis *et al.* 1976; Alley 2005). Leucocytozoonosis has also been documented in captive African (*Sphenisus demersus*) and Macaroni (*Eudyptes chrysolophus*) penguins (Peirce *et al.* 2005), and has had significant impacts on domestic poultry (*L. caulleryi*, *L. smithi*) and waterfowl (*L. simondi*) worldwide (Herman *et al.* 1975; Fallis and Desser 1977; Atkinson and Van Riper III 1991; Greiner and Ritchie 1994).

Infections range from subclinical to fatal. Signs of *Leucocytozoon* sp. infection include weakness, anaemia and sudden death (Atkinson and Van Riper III 1991). Other signs include tachypnea, petechial haemorrhage, diarrhoea, biliverdinuria, and neurological signs (Greiner and Ritchie 1994). Flock infections can manifest as seasonal or sporadic epizootic mortalities (Atkinson and Van Riper III 1991). Juveniles and immunosuppressed adults are most susceptible. Clinical leucocytozoonosis of pathogenic species is thought to be caused by a combination of anti-erythrocytic factors, gametocytes blocking capillaries, and megaloschizonts causing multifocal necrosis in a variety of tissues (Cowan 1957; Kocan 1968; Khan and Fallis 1970; Maley and Desser 1977; Greiner and Ritchie 1994).

Infectivity has both environmental and host components including vector density, immunosuppression and prior infection (Allison *et al.* 1978; Jones and Shellam 1999). Transmission studies identified the simuliid fly *Austrosimulium ungulatum* as the sole vector in Fiordland crested penguins within their natural range (Allison *et al.* 1978). Their peak feeding period was September to November during the breeding period, coinciding with peak infection rates. Infections were not fatal, but may have predisposed infected birds to secondary disease. Heavy parasitaemias were seen in

chicks and lighter burdens in adults (Allison *et al.* 1978).

Inoculated sporozoites form schizonts within hepatocytes and macrophages, undergoing asexual reproduction until the host cell ruptures (Desser and Fallis 1967). Merozoites are released and infect erythrocytes and leucocytes, or are phagocytosed by macrophages and endothelial cells to produce large protozoal cysts (megaloschizonts) up to 80µm diameter, in the liver, spleen, brain and kidney. Re-activation of latent infections is thought to occur with immunosuppression due to stress, concurrent infections or glucocorticoid administration (Atkinson and Van Riper III 1991). In fact, the level of parasitaemia may indicate the severity of underlying diseases (Remple 2004). It is proposed that due to the marked tissue phase of *Leucocytozoon* spp. compared to *Plasmodium*, recrudescence after immunosuppression or treatment is more likely.

Clinical pathology of leucocytozoonosis is non-specific. Anaemia is common and leucopenia may be present in some cases (Maley and Desser 1977). Blood smears provide a simple and rapid diagnosis (Desser and Fallis 1968), however, Gill and Paperna (2005) found that examination of peripheral smears alone may not be sufficient to determine the presence of some *Leucocytozoon* infections. Further diagnostics include DNA amplification by polymerase chain reaction (PCR) and histopathology. Identification of tissue phases in the liver, spleen and kidney provide definitive diagnosis but are difficult to acquire antemortem. Diagnosis in wild birds is usually at post-mortem during flock mortalities.

Multiple treatment strategies have been suggested, but few have proved reliably

successful. Primaquine and chloroquine have been used to address combined *Plasmodium* / *Leucocytozoon* infections in a range of species but is not highly successful in clearing *Leucocytozoon* (Evans and Otter 1998). Other treatments have included melarsomine (Tarello 2006), quinacrine HCl (Cooper 1985) and trimethoprim/sulfamethoxazole (Remple 2004) in raptors. The use of trimethoprim and sulfamethoxazole in a peregrine falcon (*Falco peregrinus*) dramatically reduced, but did not eliminate, parasitaemia (Remple 2004). We found that a similar treatment protocol apparently resulted in an improved PCV and suppression of parasitaemia to levels that were detectable by PCR, but not blood smears. Histological evidence of gametocytes in blood vessels, however, indicated a failure to completely eliminate infection. It remains unclear what effect this treatment may have had on the tissue phase of disease but the lack of viable schizonts and megaloschizonts within tissues was noteworthy.

The patient in this case was infected with multiple haematozoa and infection with a second organism may have contributed to its clinical signs. Babesiosis is uncommon and natural infections have been documented in penguins, but little is known of their significance (Brossy *et al.* 1999). *Plasmodium* spp. are widely documented as a cause of death in captive penguins, but endemic infection in wild penguins does not necessarily cause disease (Duignan 2001). Treatment in this case was directed toward leucocytozoonosis due to the large proportion of gametocytes relative to *Plasmodium* and poor availability of therapeutics for the treatment of *Plasmodium*.

Despite what appeared to be a successful treatment, this bird died during rehabilitation. Post-mortem and histological findings did not provide a definitive

cause of death. Cholangiohepatitis and interstitial pneumonia most likely represent secondary diseases in captivity. Pulmonary disease in hospitalised seabirds is very common, however this bird did not show clinical signs and the pneumonia was localised. Renal coccidiosis has been identified in little penguins (*Eudyptula minor*) and is often an incidental finding (Rose 2005). No single lesion appeared sufficient to cause death.

This report highlights the difficulty in assessing the role of endemic infections in clinical disease and illustrates some of the obstacles facing the practitioner treating wildlife. The combination of antibiotic therapy and supportive care may have had multiple beneficial effects including suppressing concurrent bacterial infection. It is likely that suppression of the parasitaemia during treatment was a factor in the initial improvement in the penguin's condition but it appears unlikely that these drugs are able to cure a patient of leucocytozoonosis. However, with wide availability and good margin of safety, their use can be advocated as an aid in recovery from this infection.

Acknowledgements

We are grateful to Maurice Alley and Laryssa Howe for their assistance with the histology and PCR applied to this case.

Table 1. Haematological and serum biochemical values

	8th Nov2006	18th Nov 2006	15th Dec 2006	Reference range	Units
WBC	9.1	14.7	6.3	1.6 – 25.1	$x10^9$/L
Lymphocytes %	25	24	36		
Lymphocytes abs.	2.3	3.5	2.3	0.61 - 14	$x10^9$/L
Monocytes %	0	3	2		
Monocytes abs	0	0.4	0.1	0.016 - 2	$x10^9$/L
Eosinophils %	0	2	3		
Eosinophils abs	0	0.3	0.2	0.05 - 2.65	$x10^9$/L
Basophils %	1	2	4		
Basophils abs	0.1	0.3	0.3	0.03 - 9.59	$x10^9$/L
Heterophils %	74	69	55		
Heterophils abs	6.7	10.1	3.5	0.61-18.8	$x10^9$/L
Haematocrit	17		31	35 – 59	%
CPK	1168			150 - 2311	IU/L
AST	695			59 - 828	IU/L
ALT	95			10 - 245	IU/L
ALP	90			0 – 237	IU/L
Total protein	38			29 – 64	g/L
Phosphorus	1.4			0.1 - 3.4	mmol/L
Calcium	2.43			1.8 - 3.1	mmol/L
Glucose	11.7			9.4 - 20	mmol/L
Uric acid	1113			101 - 1886	µmol/L

Reference ranges taken from published data on the rockhopper penguin (*Eudyptes crestatus*) - International Species Information System, March 2002

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Chapter 5

An investigation of *Leucocytozoon* in the endangered yellow-eyed penguin (*Megadyptes antipodes*): Discussion

Abstract

Leucocytozoon was identified in yellow-eyed penguin adults and chicks on Stewart Island, where it is associated with chick mortality. *Leucocytozoon* infection was limited to Stewart Island, however historical cases and statistical analysis suggest that it is likely to be present at a low prevalence on Codfish Island. Infection with *Leucocytozoon* in yellow-eyed penguins caused the formation of megaloschizonts up to 440µm diameter with a predilection for the liver and spleen, and produced round gametocytes. Clinical signs include anaemia, loss of body condition, subcutaneous ecchymotic haemorrhages and sudden death. Molecular sequencing and histology results suggest that *Leucocytozoon* in yellow-eyed penguins and *L. tawaki* in Fiordland crested penguins are difference species. Recommendations regarding future management and further investigation to determine the role of *Leucocytozoon* in yellow-eyed penguin mortality are discussed.

Introduction

The impetus for this study was the occurrence of periodic, population-wide episodes of high mortality of yellow-eyed penguins, however the results of my study describe a pathogen associated with a strong regional influence on Stewart Island. My results did not identify *Leucocytozoon* infection on the South Island and suggest is unlikely

to have been implicated in reports of general population declines observed there (Gill and Darby 1993; McKinley 2001; Moore 2001). The presence of *Leucocytozoon* sp. exclusively on Stewart Island correlated with a regional pattern of annual chick mortality where its implications on population growth may be significant.

In this chapter, I will discuss in further detail the close association between *Leucocytozoon* and chick mortality; the regional occurrence of infections on Stewart Island; the structural and molecular findings that provide new details of a *Leucocytozoon* sp. whose ecology, interactions and role in disease are unclear; and finally, the management concerns of local population decline in the world's rarest penguin species.

1. *Leucocytozoon* and Stewart Island

Leucocytozoon is a host dependent, vector-borne parasite whose range is determined by the spread of both its vectors (Simuliidae) and host. Simuliids are plentiful along the West Coast of the South Island and Stewart Island, coinciding with the nesting regions of Fiordland crested and yellow-eyed penguins, and with the presence of *Leucocytozoon*. The absence of *Leucocytozoon* infection in nesting regions of the South Island's eastern coast may reflect a less concentrated vector distribution or a scarcity of infected, dispersing penguins to introduce or maintain infection in the region.

The distribution of *Leucocytozoon* may be related to the dispersal patterns of juvenile penguins moving between breeding locations. If penguins act as reservoir hosts,

carrying infection between season and location, then the true distribution of *Leucocytozoon* is determined by the movement of infected hosts in the presence of suitable vectors. Vagrant juveniles are therefore likely to represent the key factor in *Leucocytozoon* spread. While my survey (Chapter 2) did not detect *Leucocytozoon* on the South Island or Codfish Island, the absence of detectable infection may have resulted from the limited number of penguins sampled or the absence of vagrants sampled. Thus the sampling of adults best provides an assessment of the reservoir of infection, while testing juveniles is a better measure of monitoring spread.

To further investigate this hypothesis, correlation of *Leucocytozoon* infection with the origin of birds, using banding or transponder numbers, could provide valuable insight into the movement and prevalence of *Leucocytozoon*. One vagrant yellow-eyed penguin sampled on Stewart Island with detectable *Leucocytozoon* by PCR had travelled from the South Island, however it is unclear if infection took place on Stewart Island or prior to arrival. The current prevalence data suggests infection would most likely have occurred on Stewart Island, however knowledge of prior infection at other locations through the monitoring of vagrants would be highly informative.

The unique biology of the yellow-eyed penguin makes them particularly susceptible to *Leucocytozoon* infection. Predisposing factors for yellow-eyed penguins may include the following: long nesting and fledging periods; exposure of chicks to vectors from hatching; site fidelity to land-based habitat with short-term foraging at sea; prolonged moulting periods confined to land; and a temperate nesting preference within areas of high vector density such as Stewart Island (Darby and Seddon 1990;

Moore 1992; Croxall and Davis 1999; Jones and Shellam 1999). As *Leucocytozoon* is successful in evading the host immune system, protection is best achieved by the host successfully avoiding vectors, and thereby infection (Mendes *et al.* 2005), a luxury not afforded by the lifestyle of the yellow-eyed penguin.

The role of geography in the epidemiology of *Leucocytozoon* is not well understood. Stewart Island lies between Codfish Island, the subantarctic islands and the South Island, and may support dispersing juveniles from each of these yellow-eyed penguin populations, carrying pathogens such as *Leucocytozoon* with them. Further studies that use physical and genetic identification to establish the origin of the Stewart Island birds is required to validate this hypothesis.

The disparity in *Leucocytozoon* prevalence in yellow-eyed penguins on Codfish Island and Stewart Island requires further investigation as it correlates with a difference in chick mortality. It is possible that infections with *Leucocytozoon* on Codfish Island originate from dispersing Stewart Island juveniles, with ongoing transmission being limited by a smaller host reservoir. Differences in host susceptibility between locations caused by concurrent infection, nutrition or genetic factors, may affect the ability of each population to maintain infection. Alternatively, nesting structure, colony location, and the availability of simuliid breeding habitat may influence simuliid accumulation and *Leucocytozoon* transmission. Some future avenues of research which could aid in understanding this disparity may be to examine the prevalence of *Leucocytozoon* infection in yellow-eyed penguins on the Bravo Islands, whose fledging success is similar to those on Codfish Island, and to further investigate the correlation with penguin and simuliid ecology (King 2007).

Stewart Island appears to be the main point of contact between yellow-eyed and Fiordland crested penguins, where these species are observed nesting nearby each other and using the same beach access. If *Leucocytozoon* is transmissible between penguin species, cross-infections are likely to take place among the Stewart Island penguins. Transmission in this manner may elicit different host responses, causing a different expression of disease. The extent of overlap and interaction of *Leucocytozoon* strains is currently unknown, and further examination of *Leucocytozoon* in the Fiordland crested penguins of Stewart Island may provide more detail of its impact in both species.

My survey examined the *Leucocytozoon* status of yellow-eyed penguins nesting along the northern coast of Stewart Island, but it is unclear how accurately these results reflect the total Stewart Island population. Examination of yellow-eyed penguins in the Bravo Islands and Pegasus Bay (in Southern Stewart Island) may provide a more complete knowledge of chick survival and *Leucocytozoon* infection on Stewart Island. In addition, investigation of yellow-eyed penguins nesting on Bench Island, very near to the Anglem coast but prohibited thus far by limited accessibility, may provide a direct comparison of infection and mortality in penguins nesting in the same geographical region but different habitat.

2. *Leucocytozoon* and penguin deaths

Chick mortalities on Stewart Island in 2006/07 appear to have been multifactorial. Factors under consideration which may have contributed to the overwhelming mortality include a high incidence of parental trauma, diphtheritic stomatitis,

Leucocytozoon infection, and variable levels of starvation (King 2007). The role and interactions of *Leucocytozoon* infection within this environment are presently unclear, but its association with decreased chick survival suggests its impact is significant. The potential for both primary and secondary roles in mortality for each factor will be discussed, however further knowledge is required to determine their interactions over multiple seasons.

The high incidence of parental trauma is thought to reflect misparenting by adults, or the inability of chicks to avoid being accidentally squashed. Trauma is unusual in experienced adult pairs but may occur with stress or disruption, such as simuliid harassment, human intervention or the threat of predation. Many chicks observed in this study were weak and recumbent prior to death, and may have been unable to avoid being squashed in the nest by adults. Whether multiple factors are involved in causing stress to parents and weakness in chicks is yet to be confirmed, but may be furthered by observational studies.

Diphtheritic stomatitis was identified more frequently than *Leucocytozoon* in yellow-eyed penguins, with affected chicks displaying weakness and poor growth in addition to caseous oral plaques. The incidence of diphtheritic stomatitis did not consistently correspond to the distribution of deaths by time or age, suggesting a different mechanism of disease or pattern of susceptibility compared to *Leucocytozoon*. Diphtheritic stomatitis may play a primary role, or be a predisposing factor in weakening chicks and increasing susceptibility to concurrent infection. As with other factors, the current level of knowledge creates difficulties in establishing primary

versus contributing causes of mortality, and the interaction of diphtheritic stomatitis and *Leucocytozoon* remains unknown.

There are problems with attributing high levels of chick mortality to starvation. The optimal body condition observed in adult yellow-eyed penguins makes a simple lack of available nutrients unlikely. Possible explanations include chicks failing to receive nutrition due to incorrect food presentation (misparenting); chicks being unable to properly receive parental feeding (diphtheritic stomatitis or weakness); and chicks which were unable to properly digest food (enteropathy). Evidence of maldigestion was not observed at post-mortem and diarrhoea or regurgitation were not observed in nests. Increased energy demand caused by hypothermia or mounting an immune response may produce poor development despite normal energy intake and nutrient absorption. Weight loss or reduced body condition due to haemoparasitism has been recorded in many non-penguin species and is usually most prominent in juveniles (Atkinson and Van Riper III 1991; Merino *et al.* 2000). Further evidence of the provision of food by parents and activity by chicks is required for nutrient intake and expenditure to be properly considered in chick mortality.

The influence of climate has been proposed as a primary or compounding factor on chick mortality as changing ocean temperatures affect food supply, but has not been conclusively linked to deaths on Stewart Island. The 2006 breeding season coincided with an El Nino event which occurred from July 2006 to February 2007 (Salinger and Griffiths 2007). Moore *et al.* (1991) proposed that El Nino-Southern Oscillation events may contribute to mortalities, however Peacock *et al.* (2000) found that long-term climate change was more significant than El Nino events in overall penguin

decline. Richdale (1957) described increased fledging success with warmer air temperatures and decreased success with higher rainfall, a relationship suggesting that increasing energy expenditure by chicks during poor weather conditions may be significant in addition to reduced prey availability. It is unclear why penguins on the Anglem coast would be affected by climatic variation when those on the Bravo Islands and Codfish Island did not suffer similar mortalities, and why ongoing losses occur outside of El Nino periods, suggesting that climate is a secondary influence on chick deaths. Further investigation into the association of climate and mortality at each site would be useful to determine the true impact of weather on penguin survival.

The role of *Leucocytozoon* in yellow-eyed penguin chick mortality is speculated to arise through a combination of weakness, anaemia and immunosuppression. In a primary role, anaemia and disseminated intravascular coagulation may be sufficient to cause death, while weakness and immunosuppression may predispose chicks to parental trauma, hypothermia and infection. Although the potential roles of *Leucocytozoon* have been outlined in this study, further work is needed to explore the true mechanism causing deaths in this case.

The association of age at death and *Leucocytozoon* infection requires further examination with time of initial infection, prepatent period and histological identification in neonatal chicks being vital to an understanding of *Leucocytozoon*'s role. Deaths in young chicks occurred rapidly and were clustered around 10-12 days of age, while the oldest chick died acutely prior to fledging. While disseminated megaloschizont formation in tissues and ecchymotic haemorrhage have been observed in older chicks (24-108 days), they have not been observed in neonatal chicks dying

during what appears to be the most susceptible period (10-12 days old). We assume that initial infection occurs soon after hatching due to the prolific simuliid aggregations around nests, so that infection would have to develop rapidly. *L. marchouxi*, a pathogenic species with a similar structure and life-cycle to *Leucocytozoon* in yellow-eyed penguins, has a prepatent period of 4-5 days with megaloschizonts present from 6-8 days (Peirce *et al.* 1997). If these aspects of development are similar, yellow-eyed penguins could experience clinical effects at approximately 10 days old, consistent with our clinical findings. Sampling during the prepatent period may lead to failure of detection in recently infected juveniles (Swinnerton *et al.* 2005). Knowledge of these aspects of development are important in establishing the epidemiology of infection in addition to the mechanisms of disease.

Generalised pallor was a common finding in young chicks, but its cause remains unclear. Kocan (1968) found that anaemia caused by pathogenic *L. simondi* in ducks was more severe than could be explained by destruction of parasitised erythrocytes, and proposed that intravascular haemolysis was being caused by anti-erythrocytic factors. The absence of haemosiderin deposition in the liver and spleen of affected yellow-eyed penguin chicks to indicate haemolysis, however, suggests that anaemia in these chicks may not be attributable solely to anti-erythrocytic factors, but may involve a loss of erythrocyte production. Further, erythrophagcytosis was not a prominent feature in splenic tissue examined suggesting that extravascular haemolysis due to removal of parasitised erythrocytes was also less likely as a cause of anaemia. Extramedullary haematopoiesis also showed varied responses to anaemia between cases, but is likely to be a normal response rather than characteristic of disease.

Determination between regenerative versus non-regenerative anaemia, and blood loss versus haemolytic anaemia will be significant in establishing the mechanism of mortality.

Ecchymotic haemorrhage was observed in older penguin chicks which died prior to fledging, including one 108 day old penguin where ecchymoses were observed on the feet and legs a few days prior to death (pers comm. Sandy King). Ecchymotic haemorrhage may result from loss of endothelial integrity, acute blood vessel damage and coagulopathy. Peripheral oedema was observed in younger chicks which died up to 20 days old and may be caused by increased vascular permeability, increased blood pressure, or result from hypoalbuminaemia associated with blood loss or hepatopathy. Damage caused by circulating merozoites, endothelial megaloschizonts, or release of factors involved in entry to endothelial cells, may result in widespread disruption of endothelial cell membranes and disseminated intravascular coagulation. Histological evidence of hepatopathy in chicks or endothelial cell damage in juveniles was not observed in our samples, but would be required to confirm this theory.

Further diagnostics investigating the mechanisms of death are indicated to determine the role of *Leucocytozoon* in mortalities. Characterisation of anaemia in chicks may allow differentiation between blood loss due to simuliid feeding, haemolysis through haemoparasitism, and lack of production due to chronic illness or bone marrow disease. Further testing may include more comprehensive haematology or serial sampling, bone marrow examination and serum biochemistry. Biochemistry profiles assessing protein and albumin levels, and hepatic and renal function may also be useful in determining the cause of peripheral oedema in younger chicks.

The association reported here between *Leucocytozoon* and mortality is uncommon among *Leucocytozoon* species, but not without precedent (Atkinson and Van Riper III 1991). Loss of condition and predator avoidance, and decreased foraging and immune function are described as indirect mechanisms of morbidity, while direct mortality caused by anaemia and tissue destruction is strongly associated with megaloschizont development, consistent with previous observations in other pathogenic strains (Fallis *et al.* 1974; Herman *et al.* 1975; Dawson and Bortolotti 2000; Merino *et al.* 2000; Bunbury *et al.* 2007). Variation in mortality rates between species (Khan 1968) and location (Karstad 1965) among other birds suggests that strain differences and host susceptibility may have a role in different responses between breeding groups and species of penguins. *Leucocytozoon* may have a significant role in the survival and development of yellow-eyed penguin chicks, however its activity as a primary or secondary pathogen requires further knowledge to determine, as do its interactions with the other factors outlined here. This vital information may be pursued through experimental infection, or close observation in the field over multiple seasons.

3. Simuliids and Penguins

Superficial punctate dermatitis and active simuliid haematophagia were observed by the author on yellow-eyed penguin chicks on Stewart Island, but not elsewhere. Black fly irritation and haematophagia causing nestling mortality has been described in red-tailed hawks (*Buteo jamaicensis*), great horned owls (*Bubo virginianus*) and common gulls (*Larus canus*) (Hunter *et al.* 1997; Smith *et al.* 1998; Bukacinski and Bukacinska 2000). This phenomenon, known as “Blackfly fever” causing

hyperthermia, weight loss, weakness, decreased body condition and death, has been observed in the mortalities of livestock including turkeys and chickens (Steelman 1976; Harwood and James 1979; Bukacinski and Bukacinska 2000).

Simuliid harassment of common gulls (*Larus canus*) resulted in decreased feeding frequency, and increased nest avoidance and abandonment by adults. Chick survival decreased by 45-60% in the first week after hatching, featuring dehydration and anaemia as primary causes rather than starvation (Bukacinski and Bukacinska 2000). Hunter *et al.* (1997) found that blackfly feeding alone and in concert with *Leucocytozoon* spp. (as determined by peripheral smears) caused mortality in fledgling horned owls which developed anaemia, dehydration and dermatitis. Mortality due to blackflies alone affected chicks that were less than 10 weeks old (Hunter *et al.* 1997).

Adult red-tailed hawks were unaffected by blackflies, but mortality in 14% of chicks was observed due to leucocytozoonosis and anaemia associated with haematophagia (Smith *et al.* 1998). Smith *et al.* (1998) proposed that morbidity and mortality was caused by the combined effect of early energy expenditure due to blackfly harassment, blood-feeding causing anaemia and dehydration, and *Leucocytozoon* infection causing anaemia and organ damage. Birds were considered most susceptible to blackfly bites and *Leucocytozoon* when younger than 25 days, prior to the growth of adult feather cover and maturation of the immune system which prevents burdens in older birds (Smith *et al.* 1998). We found that deaths in yellow-eyed penguin chicks occurred during this susceptible period at less than 25 days, although one case of severe leucocytozoonosis was seen at 108 days.

Physical harassment by flies may contribute to poor food delivery and increased parental trauma (Bukacinski and Bukacinska 2000). Feeding frequency in yellow-eyed penguins is determined by the change-over routine between parents approximately every 12 hours after hatching, and is unlikely to be affected by simuliid burdens. Food delivery however, may be affected, as we observed regurgitation of one parent prior to reaching the nest which may have been caused by blackfly harassment. The incidence of parental trauma observed in the 2006/07 season was higher than previous years and considered a significant contribution to hatchling deaths (King 2007). Crusting punctate dermatitis was noted among all downy chicks examined on Stewart Island and is a consistent feature of blackfly irritation in other mortalities (Hunter *et al.* 1997; Smith *et al.* 1998; Bukacinski and Bukacinska 2000).

The influence of the 1996 El Nino-Southern Oscillation event on simuliid density at Stewart Island is unknown, and current assessments of very high simuliid concentrations are subjective (Salinger 2007). El Nino events have been reported to stimulate excessive simuliid numbers (Cilek and Schaediger 2004). Bukacinski and Bukacinska (2000) postulated that the recent and progressive occurrence of massive blackfly outbreaks in Europe was related to increasing temperatures favouring blackfly reproduction. Interestingly, Peacock *et al.* (2000) found that yellow-eyed penguin fledgling success was higher during slightly cooler seasons, fitting with this indirect blackfly effect.

The direct effects of simuliids on yellow-eyed penguin chicks, including increased energy expenditure; parental trauma; decreased food delivery; anaemia; and dermatitis should be considered as significant contributors to mortality. These factors

are likely to cause or contribute to decreased growth and plumage development, starvation, weakness and increased susceptibility to disease. Evidence of these effects in yellow-eyed penguin chicks may arise from comparison of *Leucocytozoon*-free chicks in simuliid and simuliid-free areas, and investigation of the anaemia resulting from simuliid burdens. Determining the impact of simuliid harassment in the absence of their ability to spread *Leucocytozoon* will allow management to be directed toward the more significant process, the vector or the disease.

4. Morphology

The morphological identification of *Leucocytozoon* in yellow-eyed penguins is critical to our understanding of its pathogenicity, life-cycle, and relationship to neighbouring *Leucocytozoon* species. Comparison of *Leucocytozoon* spp. in yellow-eyed and Fiordland crested penguins is significant in determining the origin, spread and potential reservoirs for infection in both species. Identification also allows further research of its interaction with the host, monitoring techniques, treatment and preventative management.

The endogenous morphology of *Leucocytozoon* in yellow-eyed penguins was similar to that of *L. simondi*, with the development of megaloschizonts in the liver, spleen and other tissues. When combined with the additional finding of exclusively round gametocytes, our description becomes more consistent with *L. sakharoffi*, *L. podargii* and *L. marchouxi* (Fallis *et al.* 1974; Peirce *et al.* 1997; Adlard *et al.* 2002). Of these similar species, both *L. simondi* and *L. marchouxi* are associated with mortality in free-ranging populations (Fallis *et al.* 1974; Peirce *et al.* 1997; Bunbury *et al.* 2007).

The morphology of *Leucocytozoon* infection in yellow-eyed and Fiordland crested penguins was dissimilar, sharing only the presence of round gametocytes (Fallis *et al.* 1976; Allison *et al.* 1978). Infections in yellow-eyed penguins were characterised by the presence of widespread megaloschizonts and rare primary schizonts, findings which are not consistent with infections in Fiordland crested penguins (Allison *et al.* 1978). Infections caused by one *Leucocytozoon sp.* may demonstrate dissimilar development and clinical signs in different hosts, making accurate species identification by morphology and molecular techniques important to the management of infections.

A similar situation was faced by Desser *et al.* (1978) in Michigan, USA, when strain differences in *Leucocytozoon* were first identified. Desser *et al.* (1978) found that *L. simondi* in Canada goslings (*Branta canadensis*) produced disseminated megaloschizont formation and mortality at Seney National Wildlife Refuge, while *L. simondi* infections in populations 40km and 278km away produced primary schizonts and round gametocytes without signs of illness. The restricted or 'partial development' in one region compared to another suggested geographic-associated variation in *L. simondi* pathogenicity, and has resonance in the comparison of infections in penguins on Stewart Island and in Fiordland (Desser *et al.* 1978). These comparisons, made before molecular techniques to distinguish haematozoa were available, could not provide information on the genetic relationship of the 'complete' and 'partial' strains.

5. Molecular findings

The identification of *Leucocytozoon* sp. DNA by PCR and sequencing allows differentiation at the genetic level. Positive results indicate the presence of the organism but do not discriminate between life stages of the parasite, such that sporozoites and merozoites, gametocytes and megaloschizonts are not distinguished. In cases where infection was not also investigated using histology, we could not determine if infections were in the initial or mature stage of development. This has significant implications for our attempts to establish the role of *Leucocytozoon* infection in mortality. Positive results incurred through the detection of sporozoites may represent recently infected animals which could subsequently clear infection. Detection of infection by PCR remains useful in identifying the presence of *Leucocytozoon*, allowing the determination of prevalence, and defining the molecular characteristics of the *Leucocytozoon* species involved.

The use of PCR in this study allowed the identification of infections at lower intensities than could be observed using blood smears. This finding is consistent with other studies surveying low-level parasitaemias (Jarvi *et al.* 2002; Richard *et al.* 2002; Swinnerton *et al.* 2005). Infection with other haemoparasites was not observed on blood smears so confusion with other haematozoa species was unlikely, but may be possible at low levels (Valkiunas *et al.* 2006).

Leucocytozoon sp. DNA from adult and juvenile yellow-eyed penguins showed strong similarity, suggesting the possibility that *Leucocytozoon* is being transmitted from a reservoir of chronically infected adults to their chicks. Chernin (1952) found that *L.*

simondi infection in adult ducks relapsed with the onset of reproductive behaviour in spring, with increased adult parasitaemias promoting transmission to chicks during the early breeding season. Chronic infections in adults have significant implications for management as eradication is not immediately feasible and invasive actions to prevent transmission may result in relapse (Atkinson and Van Riper III 1991).

Sequences of *Leucocytozoon* sp. DNA from yellow-eyed penguins on Stewart Island and two Fiordland penguins found in the Foveaux Strait, 39km further north, were more distantly related, indicating that these Fiordland crested penguins were not infected with the same species of *Leucocytozoon* as yellow-eyed penguins on Stewart Island (Fig 2, Chapter 2). This differentiation supports the histological findings in which morphology was also distinct between species. Histology from one Fiordland crested penguin was characteristic of *L. tawaki* in this species.

Variation was also seen between the two *Leucocytozon* sp. sequences from the Fiordland crested penguins. This variation may reflect the geographical separation in each penguin's origin; a high susceptibility to genetic variation within *L. tawaki*; or a subset of species within what is currently termed *L. tawaki* at the molecular level. Further investigation is required to determine the variability of *L. tawaki* within the Fiordland crested penguin population, and establish sequence data for future investigation. On the basis of molecular identity, the Fiordland crested penguin vagrants tested in this study were not likely to have been infected on the Anglem coast of Stewart Island by *Leucocytozoon* from yellow-eyed penguins, although the closer similarity of one *L. tawaki* sequence to those on Stewart Island may indicate a closer geographic association.

If interspecies transfer occurs with the ability to cause pathology, the finding of a vagrant Fiordland crested penguin with clinical leucocytozoonosis in Auckland indicates the potential range of distribution (see chapter 4). While *Leucocytozoon* has been documented in Fiordland crested penguins, investigation of the clinical and population effects have not been pursued. Further work is needed to determine the prevalence, characterisation and clinical effects of *Leucocytozoon* in Fiordland crested penguins.

6. Taxonomy

Leucocytozoon species are commonly named according to the host species in which they were first discovered (Desser and Bennett 1993). This relies on the assumption that species are strongly host-specific at the family level (Desser and Bennett 1993; Peirce 2005a). *Leucocytozoon* found in Macaroni and African penguins with similar morphology to those first described in the Fiordland crested penguin are therefore referred to as *L. tawaki*[♣] (Allison *et al.* 1978; Earle *et al.* 1992; Peirce 2005b). Allison *et al.* (1978) found that *L. tawaki* was transmissible from Fiordland crested to Little penguins (*Eudyptula minor*) so this distinction appears valid. What is not apparent is the behaviour of *Leucocytozoon* in different host species, and whether multiple *Leucocytozoon* spp. exist within the family Spheniscidae.

The second distinction is made on the basis of gametocyte morphology in peripheral blood, and is the primary means of species identification (Desser and Bennett 1993). In one yellow-eyed penguin, gametocytes were observed in visceral tissues rather than

[♣] 'Tawaki' is the Maori name for the Fiordland crested penguin

peripheral smears, and were round rather than elongate as occurs during the megaloschizont stage of other *Leucocytozoon* spp. (Fallis *et al.* 1974). There are a growing number of *Leucocytozoon* spp. demonstrating exclusively round gametocytes in combination with a megaloschizont life-cycle, but distinctions between *Leucocytozoon* species using these characteristics in the literature are few (Peirce *et al.* 1997; Adlard *et al.* 2002).

We found that differences in the endogenous life-cycle, morphology and molecular sequences exist between the *Leucocytozoon* spp. found in yellow-eyed penguin on Stewart Island and *L. tawaki* from Fiordland crested penguins. The naming of novel *Leucocytozoon* infections on the basis of tissue phases and molecular differences appears well founded, but precedents are lacking for species distinction without the identification of gametocytes in peripheral blood.

7. Risk analysis

There is an inherent risk in endemic infections that alteration of the host-parasite-environment relationship will lead to changes in disease expression. For vector-borne diseases this includes the added consideration of disease spread in response to changes in vector ecology. Changes to the host such as increased baseline stress, concurrent disease, and increased energy expenditure in reproduction or foraging may increase the likelihood of *Leucocytozoon* recrudescence with associated morbidity, and increased transmission from the resulting parasitaemias (Chernin 1952; Khan and Fallis 1970; Cranfield *et al.* 1994). Fortunately, the opposite is also true, with changes in favour of the host, such as reduced stress or shelter from vectors, having

the potential to decrease clinical effects and minimise or prevent the expression of disease.

Climate change and habitat availability have significant impacts mediated through vectors (Dobson and Carper 1992). Increased vector presence on the South Island with altered climate confers an increased likelihood of *Leucocytozoon* transmission by the arrival of infected vagrants, even where their presence in previous seasons was apparently benign. Enhanced vector populations also increase *Leucocytozoon* exposure of chicks and adults, raising the dose-dependant pressure of infection.

Habitat alteration, a current threatening process for yellow-eyed penguins on the South Island, also has implications for their interaction with *Leucocytozoon*. This includes the availability of breeding sites for penguins and simuliids; availability of cover for nesting penguins to escape vectors; extent and intensity of vector loading; and the direct and indirect effects of environment on host immune function. Energy requirements to meet foraging, reproductive, maintenance and immune demands can easily become unbalanced when food shortage, prolonged moulting, concurrent disease or stress is applied.

Within this changing framework it is recommended that monitoring for the presence of *Leucocytozoon* along the South Island coastline be continued, so we may be aware of the alterations in disease interactions with environmental ones. The risk to the subantarctic islands, which houses the bulk of the yellow-eyed penguin population, is dependant on vector availability, and should be closely examined using a similar study.

8. Management considerations

In light of the above risk factors, it is worth commenting on areas where management may require consideration of *Leucocytozoon* infection. It is possible that movement of yellow-eyed penguins will occur for veterinary treatment or translocations to establish new breeding sites. The concern is that unnecessary or stressful procedures such as transportation may result in recrudescence of *Leucocytozoon* infections.

At its most extreme, movement and/or treatment of yellow-eyed penguins may be required during an oil spill response involving transport and concentration of yellow-eyed penguins during treatment and rehabilitation. Such a scenario has precedence in the oil spill response managament of 20 000 penguins in South Africa in 2000, and of little blue penguins in Tasmania in 1995 (Goldsworthy *et al.* 2000; Callahan 2001). This combination of stress and increased host density may result in both recrudescence and optimised transmission of *Leucocytozoon* through vectors. Vector exclusion using netting or appropriate insecticides, stress reduction by visual barriers and minimal captive periods would likely ameliorate some of these effects, although recrudescence and parasitaemias may still occur. While blood smear examination proved a poor test for the presence of *Leucocytozoon* in our study, it may provide some information in acutely infected parasitaemic birds to assist in initial management and rehabilitation.

Translocation of infected individuals from endemically diseased populations to disease free populations in the presence of vectors should not be pursued. At this time insufficient knowledge of the range of infection and vectors, and the actual role of

Leucocytozoon in causing mortality make accurate management difficult and should therefore be approached with caution. It is also recommended that during holding or transport, yellow-eyed penguins be housed separately from other penguins and excluded from insect vectors. These considerations should not hinder the treatment of injured yellow-eyed penguins as concurrent disease or injury is likely to have far greater impact on *Leucocytozoon* infection than transport.

As captive penguin collections seek to exclude insect vectors of *Plasmodium*, vector exclusion should also be addressed in the housing of yellow-eyed penguins in temporary holdings. Secure mosquito-proof netting in addition to hygienic husbandry procedures should be sufficient for this purpose.

9. Vaccination

Vaccination may represent a potential solution to leucocytozoonosis in yellow-eyed penguins. The most promising vaccine currently available is a one dose, oil-adjuvanted vaccine which is effective for up to 5 months against *L. caulleryi* in chickens. This vaccine may provide limited cross-protection, however the limiting factor in management appears to be the post-inoculation delay until immunity, which is approximately 12-18 days (Ito and Gotanda 2004). Given that in 2006, nine of 32 (28%) yellow-eyed penguin chick deaths occurred after 2 weeks of age, vaccination soon after hatching may be a feasible strategy for preventing *Leucocytozoon* associated mortality in older chicks.

The mechanism of natural immunity to haemoparasitism in this case is unclear, but is an area which may improve the success of future preventative programs. In our study, it appeared that exposed, non-clinical adult yellow-eyed penguins did not provide immunity to chicks by passive transfer in the yolk sufficient for their protection in the first weeks of life. Additionally, Fallis *et al.* (1974) reported that birds harbouring chronic infections which were protected from vectors for prolonged periods of time often became heavily reinfected when re-exposed to *Leucocytozoon* and died. Thus he proposed that continual introduction of sporozoites was required to maintain immunity (Fallis *et al.* 1974). Trials to determine the efficacy of vaccination in yellow-eyed penguins would be critical in preventative action, but would require a committed partnership between scientists and conservation managers.

It is concerning that trials of *Leucocytozoon* vaccine have only been performed on chickens older than 5 weeks with *L. caulleryi*. Vaccination of adult chickens failed to reduce parasitaemias, making it of little use in preventing or suppressing transmission of *Leucocytozoon* from adult penguins. No evidence has yet been presented examining maternal immunity or vaccination of young chicks, and oral administration is not yet feasible due to excessive dosage requirements (Ito and Gotanda 2004). An alternative method using spleen homogenates containing the required *Leucocytozoon* sp. could provide a more targeted method, but would be difficult to produce and validate (Isobe *et al.* 1991).

Prior to administration of vaccines to yellow-eyed penguin chicks, there are a number of issues regarding the efficacy in penguins, efficacy against *Leucocytozoon* in yellow-eyed penguins, and safety in neonatal penguins that should be considered.

Choice of vaccine type, eg. recombinant vs. spleen homogenate, and timing of inoculation would also need to be carefully considered. Benefits in preventing older chick deaths must consider the risk of abandonment when disturbing nests with young chicks and increasing the exposure to vectors during the process. Finally, an appropriate monitoring system to assess efficacy, such as survival or titre levels, should be in place prior to administration.

If vaccines could be administered to chicks without causing nest abandonment or parental trauma, and *Leucocytozoon* was confirmed as a significant pathogen, a trial of the recombinant vaccine on chicks on Stewart Island would be recommended. Further research to test safety and efficacy could include vaccine trials in day old chicks of another species prior to trials in yellow-eyed penguins, and monitoring of titres at routine 20 day-old checks once a program is implemented.

10. Further research

The more we learn about our ever-diminishing native fauna, the more there seems to be to discover. Unfortunately our knowledge of the yellow-eyed and Fiordland crested penguin, probably the most endangered of the world's penguins, is lacking at even the most basic of levels. These cryptic species have evaded reliable census and much of their physiology and biology remains unknown. It is hoped that the questions arising here will help to ignite the enthusiasm and interest of researchers to apply their trade to these most endangered and wonderful creatures.

The underlying causes of ongoing population mortality in yellow-eyed penguins remain unknown. This should be a priority area of future research. The extent of mortalities on Stewart Island requires investigation and the genetic status of the population there needs to be uncovered. Following from this investigation, further determination of the role of *Leucocytozoon* infection in yellow-eyed penguins is a recommended next step in discovering the cause of chick deaths on Stewart Island and preventing further mortalities. Further afield, the prevalence of *Leucocytozoon* and presence of its vectors could be established on the subantarctic islands where the majority of the population resides.

There is considerable scope for investigation to quantify and characterise the anaemia that was observed in chicks. Investigation outside of blood smear analysis was not pursued in this study due to limitations in field sampling, time spent at nests, delay till centrifuging and equipment. Further haematological assessment including packed cell volumes and plasma protein could quantify anaemia and differentiate blood loss, such as simuliid feeding, from haemolysis or bone marrow suppression, the latter being determined by cytology or histology. Serial sampling in neonates would be difficult given the risk of nest abandonment by parents but may be achievable if samples were taken in the presence of different parents on the nest. Determination of regenerative or non-regenerative anaemia may provide further evidence to establish the mechanism of disease. Investigation of anti-erythrocytic factors as described by Kocan (1968) may also be useful, although evidence of haemolysis was not clear in this study.

Investigation into peripheral oedema and ecchymotic haemorrhage may be furthered by serum biochemistries in chicks evaluating liver function (aspartate

aminotransferase, lactate dehydrogenase, bile acids), protein production (total protein, albumin, fibrinogen) and coagulation time without the requirement for post-mortem. It should be noted that these signs occurred within 12-72hrs of death so caution in sampling and administration of supportive care such as fluid support would be advised.

Further investigation is also warranted into the primary role of biting flies in penguin morbidity through anaemia or misparenting, as has been described in other species. Evidence of reduced packed red cell volumes and plasma protein in chicks suggesting blood loss could be compared with simuliid feeding intensity to provide preliminary evidence, although the impact of concurrent haemoparasitism should also be assessed.

The phenomenon of recrudescence (seasonal or stress-induced) as a mechanism for increasing spread to chicks might be assessed by testing of adults in the breeding and non-breeding season, and there is considerable scope for the investigation of *Leucocytozoon* vaccination in penguins, although this may be limited by expense and limited subjects to validate a final product.

The identification of vectors in yellow-eyed and Fiordland crested penguins on Stewart Island may provide valuable information on the transmission of *Leucocytozoon* and the potential for inter-species transfer. For example, if *L. tawaki* occurs throughout the natural range of Fiordland crested penguins, but *Leucocytozoon* in yellow-eyed penguins is limited to those areas where yellow-eyed and Fiordland penguins interact, it is feasible that at some point infection in yellow-eyed penguins originated from a divergent *L. tawaki* strain.

In Fiordland crested penguins, the prevalence and role of *L. tawaki* could provide valuable information for both species. It is surprising and concerning that 30 years after the identification of *L. tawaki*, its effect on the host and its epidemiology within the population remains unknown.

Ultimately, the major goal of scientific investigation of our endangered species is to provide the information with which conservation managers can make informed decisions to sustain these vulnerable species through the dramatic changes we make to their environment, and those changes we will continue to make in the future.

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