Host-parasite dynamics in the endangered yellow-eyed penguin (*Megadyptes antipodes*): investigations of *Plasmodium* and *Eimeria* in geographically distinct populations

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ABSTRACT

The yellow-eyed penguin (*Megadyptes antipodes)* is an endangered species endemic to New Zealand, facing ongoing threats to the persistence of the species. The yellow-eyed penguin occupies a restricted range including mainland New Zealand and adjacent islands, and the subantarctic Auckland Islands and Campbell Island. There has been no effective migration between the birds occupying mainland New Zealand, and the subantarctic islands, creating two population centres that are both geographically and genetically distinct. The two population centres of the yellow-eyed penguin, defined by the geographical range, occupy vastly different habitat ranges and face ongoing site-specific threats. The mainland population has declined by up to 65% in the last 20 years, while the sub-Antarctic population appears stable through limited monitoring.

Examining host-pathogen dynamics of geographically distinct wildlife populations can inform predictions of a population's response to major climate shifts or translocations. This study therefore explored possible differences in the host-pathogen dynamics between the two population centres of the yellow-eyed penguin. The study sampled wild penguins from the two major population centres, the mainland and sub-Antarctic population centres, to examine the prevalence and pathogen load differences of two known parasites *Eimeria* and *Plasmodium.* In addition, the study documented differences in the prevalence of *Plasmodium* infection between penguins in rehabilitation and those in the wild.

Eimeria is a host-adapted parasite that principally causes disease in wild birds when the host is immunosuppressed through stress or intercurrent disease. Therefore, this study used *Eimeria* as an ecological biomarker to assess the distinct wild yellow-eyed penguin populations. The first morphological description of a novel species of *Eimeria* protozoa from a yellow-eyed penguin host is provided in this study. A high prevalence and pathogen load of *Eimeria* oocysts in faecal samples from wild yellow-eyed penguins was identified from the wild sub-Antarctic (apparent prevalence 76.6%, 95% CI 62.78-86.40% and mean pathogen load 9723 +/- 5831 oocysts/gram) and mainland populations surveyed (apparent prevalence 58.5%, 95% CI 43.37-72.24% and mean pathogen load 1050 +/- 398 oocysts/gram), with only weak evidence for a difference in the apparent prevalence and pathogen load between the two population centres. There was, however, a significant difference in body condition scores of infected penguins between the two populations, suggesting possible differences in the host-pathogen dynamics between the two distinct population centres. These results confirm the hypothesis that these coccidia are characteristic of a highly host-adapted endemic parasite in the yellow-eyed penguins.

Penguins have a known susceptibility to *Plasmodium* sp., with infection capable of causing significant morbidity and mortality. This study provides the first report of a positive result for *Plasmodium* sp. from wild

yellow-eyed penguins using LSU-RNA qPCR, and documents the estimated pathogen load (mean pathogen load 122 +/-29 *Plasmodium* DNA copies / 10,000 avian cells) of infected wild birds. There was good evidence for a difference in apparent prevalence between the two population centres, with a higher apparent prevalence of avian malaria in the wild mainland population (6.8%, 95% CI 2.96-15.05%), and an apparent absence of infection in the sub-Antarctic population surveyed (0%, 95% CI 0-5.58%). The study also documented a high apparent prevalence (65.9%, 95% CI 51.14-78.12%) of *Plasmodium* sp. infection in yellow-eyed penguins in rehabilitation, identifying three separate strains of avian malaria (*Plasmodium* sp. Lineage LINN1, *Plasmodium relictum* lineage SGS1 and *Plasmodium elongatum* lineage GRW06) via molecular sequencing. These results confirm the hypothesis that avian malaria is characteristic of a vector-borne parasite in the yellow-eyed penguins, and despite the presence of competent vectors in both habitats, only the northernmost population of yellow eyed penguins is currently infected. However, this disease is likely to emerge in the sub-Antarctic population in response to predicted climate shifts.

The results of this work provide a platform for further research into the host-pathogen dynamics of the novel *Eimeria* species identified, and the potential host effects of this parasite during environmental stressors. In addition, the findings of this study suggest future monitoring of avian malaria prevalence and mortality rates in the yellow-eyed penguin are critical to understand the emerging risk of this pathogen in the context of ongoing climate shifts. Finally, the high prevalence of *Plasmodium* infection documented in yellow-eyed penguins in rehabilitation demonstrates the threat of this parasite to the success of ongoing rehabilitation efforts, and the need to investigate measures to mitigate infection risk for this species in the future.

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Introduction, literature review and research aims

CHAPTER ONE - INTRODUCTION, LITERATURE REVIEW AND RESEARCH AIMS

1.1 INTRODUCTION

The yellow-eyed penguin (*Megadyptes antipodes)* is one of the most endangered species of penguin globally (BirdLIfe International, 2018) and is endemic to New Zealand. The species colonised mainland New Zealand 500 years ago, following the extinction of another penguin species *Megadyptes whaitaha* (Boessenkool *et al.*, 2009a). This northern expansion of the yellow-eyed penguin created two genetically and geographically distinct population centres, defined by the geographic location, between the Sub-Antarctic and the South Island of New Zealand (Boessenkool *et al.*, 2009a, 2009b, 2010) (Figure 1.1). Surveys of the mainland population on the South Island of New Zealand show a persistent overall population decline, with intermittent mass mortality events that may be the result of toxin exposure, infectious disease, or changes in food supply (Ellenberg and Mattern, 2012, Gartrell *et al.*, 2017, Mattern *et al.*, 2017, BirdLIfe International, 2018). The population dynamics of the Sub-Antarctic birds remain largely unknown due to the isolation of the habitat, but recent surveys suggest the overall population of the Auckland Islands appears stable despite large interannual fluctuations (Muller *et al.,* 2020a).

Geographically distinct wildlife populations may be exposed to diverse and unique challenges in host-pathogen dynamics. Examining the differences in these dynamics can inform predictions of a population's likely response to major climate shifts, or colonisation (Singer *et al.*, 2013). The two population centres of the yellow-eyed penguin, occupy vastly different habitat ranges, with the mainland birds occupying the northern-most extreme of the habitat range for the species. The geographic barrier and genetic distinction between the two populations, means that ongoing population dynamics likely differ between the two populations (Boessenkool *et al.*, 2010, Seddon *et al.*, 2013). In addition, there has been previous evidence of differing host-pathogen dynamics between the two populations (Argilla *et al.*, 2013, Argilla, 2015), and the small effective size of the South Island population make them increasingly susceptible to environmental changes and habitat stressors (Boessenkool *et al.*, 2010).

This study therefore aimed to explore the possible differences in host-pathogen dynamics between the two distinct population centres of the yellow-eyed penguin. There is evidence that parasitic relationships can exacerbate the effect of population level habitat or climate shifts (Singer *et al.*, 2013). This study examined the prevalence and pathogen load of two known parasites of yellow-eyed penguins to explore differences in the host-pathogen relationships between the two population centres.

Coccidia are obligate intracellular parasites of the phylum Apicomplexa, and infect all classes of vertebrates. Coccidia are usually known to be of low clinical significance in wild populations, rarely causing disease in the absence of stress or intercurrent disease that lowers host immunity (Alley and Gartrell, 2019). Coccidia are frequently host-adapted parasites and are often found at an easily detectable prevalence within wild populations of birds (Yabsley, 2009). *Eimeria,* a genus of coccidia, was chosen as a study pathogen to attempt to detect the potential impact of stressors on the host-pathogen dynamics of the two distinct wild population centres of yellow-eyed penguins.

Avian malaria is a mosquito-borne disease caused by intracellular protozoans of the genus *Plasmodium*. Avian malaria is an wide-spread disease of emerging importance in temperate zones due to the effects of climate change increasing the spread and incidence of disease globally (GaramszegI, 2011, Fortini *et al.*, 2020). Penguins are considered highly susceptible to avian malaria, with outbreaks of disease causing significant mortality in captive populations (Grilo *et al.*, 2016, Taunde *et al.*, 2019). Avian malaria has been reported as a sporadic cause of mortality in wild yellow-eyed penguins in the mainland population (Alley, 2001, Hunter *et al.*, 2016). However, previous surveys have failed to detect the parasite in free-living penguins of mainland New Zealand despite high seroprevalence results (Graczyk *et al.*, 1995, McDonald, 2003, Sturrock and Tompkins, 2007). In addition, the more northern population of yellow-eyed penguins on the South Island of New Zealand may be at a greater risk of avian malaria than the Sub-Antarctic population due to the known vector density on mainland New Zealand (Tompkins and Gleeson, 2006, Sturrock and Tompkins, 2008). Avian malaria therefore, was chosen as the second study pathogen to explore possible host-pathogen dynamic differences between the two population centres, and to investigate if *Plasmodium* disease dynamics could threaten the yellow-eyed penguin population overall.

So, in summary, two pathogens were selected for study, coccidia on the basis that it was likely to be a common, host-adapted parasite with low pathogenicity, and avian malaria as a vector driven parasite that is of increasing significance to yellow eyed penguin populations as a result of predicted climate shifts in both populations. What effect would the different environments occupied by the two main populations of yellow eyed penguins have on host pathogen dynamics of these parasites?

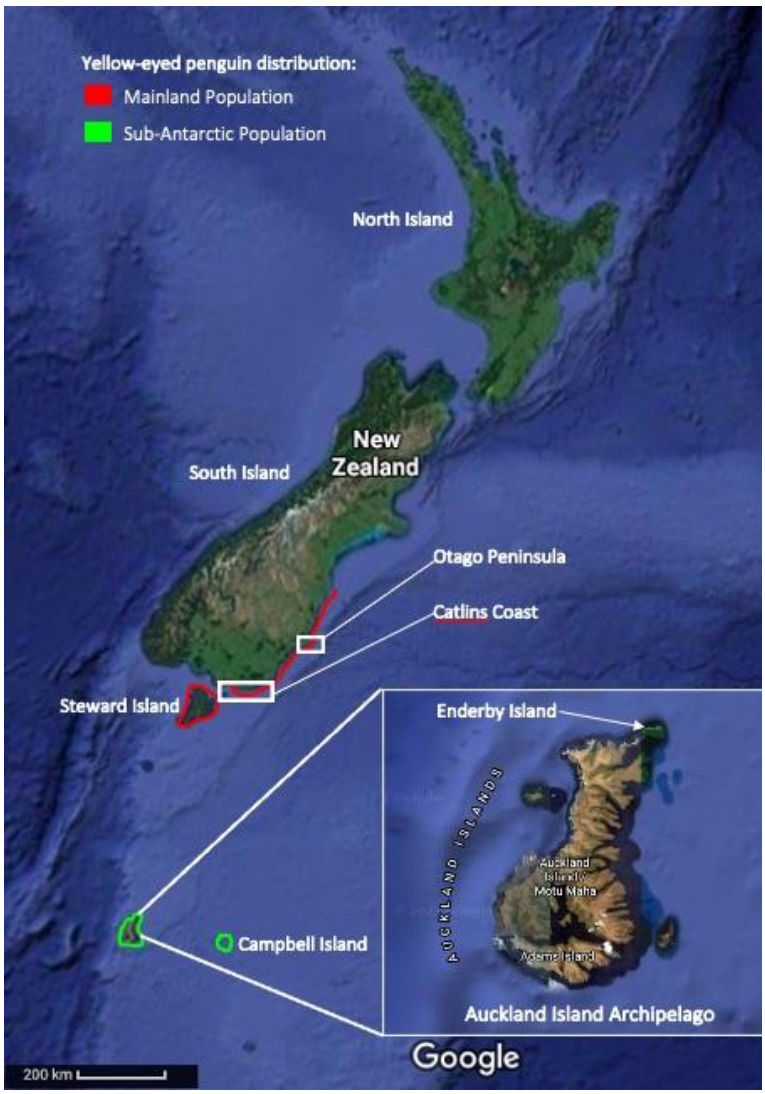

Figure 1.1 Population distribution of the yellow-eyed penguin (*Megadyptes antipodes*)

1.2 **LITERATURE** REVIEW

1.2.1 Yellow-Eyed Penguin Ecology

1.2.1.1 Conservation status, genetic diversity and migration

The yellow-eyed penguin (*Megadyptes antipodes)* is endemic to New Zealand and is one of the most endangered species of penguin globally (BirdLIfe International, 2018). The yellow-eyed penguin also known as *Hoiho*, meaning "noise-maker" (Maori), and is a tall (approximately 65 cm) and distinctive penguin with a characteristic yellow band of feathers passing around the eyes and across the nape (Seddon *et al.*, 2013). The yellow-eyed penguin population has declined over the last 30 years, with periods of extreme fluctuation and mortality (BirdLIfe International, 2018). The estimated total population consists of 1,700 breeding pairs

distributed between two main population centres consisting of the South Island of New Zealand's south east coast and the sub-Antarctic (Seddon *et al.*, 2013). The mainland populations have been studied extensively with estimates of 580-780 breeding pairs along the south east coastline and Stewart Island (McKinlay, 2001, Massaro and Blair, 2003, Seddon *et al.*, 2013). The stronghold of the population in the sub-Antarctic is believed to hold 68-79% of the population (Muller *et al.*, 2020a), with estimates ranging between 444-922 pairs on the Auckland Islands (Muller *et al.,* 2020), and 350-540 pairs on Campbell Island (Moore, 1992, 2001). A recent survey of the Auckland Islands showed that the population appears to have been stable overall through 2012-2017 despite large annual fluctuations (Muller *et al.,* 2020a). However, due to the isolation of the sub-Antarctic population, surveys have occurred infrequently, and population dynamics remain largely unknown.

Modern genetic analysis has shown that the yellow-eyed penguin recently expanded their habitat range to include the South Island 500 years ago with the extinction of another penguin species *Megadyptes whaitaha* (Boessenkool *et al.*, 2009a). In addition, this research has demonstrated that the sub-Antarctic and South island (including Stewart Island and surrounding islands) birds are genetically distinct, with minimal migration of individuals occurring between the populations. The geographic barrier and genetic distinction between the populations means they should be managed as separate populations, and population dynamics on the South Island are not necessarily mirrored in the sub-Antarctic (Boessenkool *et al.*, 2009b, 2010, Robertson, 2017). Furthermore, Boessenkool *et al.* (2010) suggested that the small effective size of the South island population means this population of yellow-eyed penguins are at particular risk of gradually losing the already low genetic diversity in the population. This lack of genetic diversity would make them increasingly susceptible to changes in environmental conditions, disease and stressors (Boessenkool *et al.*, 2010). Ongoing research and monitoring of the distinct yellow-eyed penguin populations is critical to further understanding of the population dynamics and inform conservation decisions.

1.2.1.2 Reproduction

Yellow-eyed penguins are strongly philopatric and non-colonial, choosing nest sites that are visually isolated from other penguins and concealed in dense vegetation (Seddon, 1989, Darby *et al.*, 1990). The environment available for nesting on mainland New Zealand has changed substantially due the destruction of native forest cover. Historically, yellow-eyed penguins nested in cool lowland podocarp forests, where now the mainland penguins breed in remnant shrub habitats, choosing sites to provide maximum protection from heat stress (Ellenberg and Mattern, 2012, Clark *et al.*, 2015). The penguins are present at breeding grounds year-round. Nests are constructed in September with a clutch of two eggs usually laid 3-5 days apart in late September to Early October (Seddon, 1989, Darby *et al.*, 1990, Seddon and Darby, 1990). Incubation is shared between the parents, and hatching occurs synchronously in most cases after an incubation period of 39 to 51 days (Richdale, 1957, Seddon and Darby, 1990). Chick rearing is also shared between the parents and is comprised of two phases. The first phase, the guard phase lasts 40 to 50 days, where the chick is constantly attended to at the nest. The second post guard phase describes the stage when chicks are left alone at the

nest until parents return for feeding in the late afternoon or evening (Seddon, 1989, Darby *et al.*, 1990, Seddon and Darby, 1990). Chicks fledge from mid-February to mid-March at approximately 106 days of age (Seddon *et al.*, 2013). Tremendous inter-annual variation in chick mortality and fledging weights has been described as a result of variations in food availability (Darby *et al.*, 1990, van Heezik and Davis, 1990), disease (Alley *et al.*, 2017) and suspected heat stress (Argilla, 2015; Clark *et al.*, 2015).

1.2.1.3 Diet and foraging

The foraging strategy used by wild yellow-eyed penguins appears to vary between the mainland and sub-Antarctic populations (Muller *et al.*, 2020b). Yellow-eyed penguins of the mainland population of New Zealand are highly specialised benthic feeders, and selectively feed in discrete locations over the continental shelf (Moore, 1999, Mattern *et al.*, 2007). The average foraging trip for nesting penguins is between 10 to 14 hrs, with mainly benthic diving (87% of dives) for feeding and pelagic dives for travel to and from feeding grounds (Mattern *et al.*, 2007, Chilvers *et al.*, 2014, Mattern *et al.*, 2018). The sub-Antarctic population has been recorded performing pelagic dives and foraging trips, with birds diving on average significantly deeper than the mainland population (Muller *et al.*, 2020b). In addition, yellow-eyed penguins of the sub-Antarctic demonstrated diving plasticity with individuals using both benthic and pelagic foraging strategies (Muller *et al.*, 2020b). Seven species of fish constitute 95% of the diet of birds on the South Island including opalfish (*Hemerocoetes* sp.*),* blue cod (*Parapercis colias)*, sprat (*Sprattus antipodum)*, red cod (*Pseudophycis bachus),* ahuru (*Auchenoceros punctatusi)*, silverside *(Argentina elongate)* and arrow squid *(Nototodarus* sp.*)* (van Heezik, 1990, Moore and Wakelin, 1997, Browne *et al.*, 2011). Significant variation in meal sizes and species composition has been demonstrated between different habitat localities and between seasons (van Heezik, 1990, Moore and Wakelin, 1997, Browne *et al.*, 2011). Yellow-eyed penguins of the mainland population show high individual site fidelity with feeding locations, making very consistent foraging trips. Most penguins of the South Island are mid-shelf foragers (5-16 km off the coast), however there is great inter-individual variation between chosen foraging sites (Moore, 1999, Mattern *et al.*, 2007). The penguins of the sub-Antarctic population show greater foraging plasticity than the mainland birds, which may be due to improved foraging conditions, and access to a greater vertical area for foraging (Muller *et al.*, 2020b). The selective benthic foraging strategy of the yellow-eyed penguin of mainland New Zealand makes them particularly vulnerable to variations in prey availability and changes to the continental shelf environment.

1.2.1.4 Threats

The yellow-eyed penguin populations of mainland New Zealand and the sub-Antarctic are susceptible to both marine and terrestrial threats. Habitat degradation has had the greatest impact on the mainland population in the terrestrial environment (Seddon *et al.*, 2013). Alterations to the breeding habitat, including the loss and fragmentation of habitat, has reduced the reproductive success of mainland birds through the

effects of predation, human disturbance, disease and heat stress (Seddon and Darby, 1990, McKay *et al.*, 1999, Moore, 2001, Alley *et al.*, 2004, Seddon *et al.*, 2013). Introduced terrestrial predators are a significant challenge for all native New Zealand wildlife, including the yellow-eyed penguin. Predation, particularly of chicks, by uncontrolled dogs (*Canis lupus familiaris)*, introduced ferrets (*Mustela furo)* and stoats (*Mustela erminea)* and possibly feral cats (*Felis catus)* has had a significant impact on mainland populations (Darby *et al.*, 1990, Alterio and Moller, 1997, Ratz and Murphy, 1999, Alley *et al.*, 2004, Ratz *et al.*, 2004, Hocken, 2005). Feral cats (*Felis catus)* and pigs (*Sus scrofa)* are present on the main Auckland Island and are suspected predators of the sub-Antarctic birds (Moore, 1992). In addition, predation by New Zealand sea lions has been a documented cause of local population declines in the sub-Antarctic population (Moore and Moffat, 1992, Moore, 2001). Human disturbance by unregulated tourists at mainland breeding sites has been associated with decreased fledging weights and decreased reproductive success in the mainland birds (McClung *et al.*, 2004, Ellenberg *et al.*, 2007).

Yellow-eyed penguins of the mainland and sub-Antarctic populations are vulnerable to alterations in their marine habitat (Muller *et al.,* 2020b). One of the most significant marine threats to the population overall is the marked inter-annual variation in food supply (Seddon *et al.*, 2013). Inadequate or poor-quality food supply has been associated with high chick mortality, starvation and mortality in juveniles and adults, and poor reproductive performance (Darby *et al.*, 1990, van Heezik and Davis, 1990, Alley *et al.*, 2004, Browne *et al.*, 2011, King *et al.*, 2012). Modelling has suggested improved fledging success in cooler and wetter seasons which may be related to temperature effects on fish stocks (Peacock *et al.*, 2000) or direct heat stress in the chicks (Argilla, 2015). In addition, increases in sea surface temperature due to climate change are projected to cause decreased productivity and survival of the yellow-eyed penguin (Mattern *et al.*, 2017). Mattern *et al.* (2017) attributed 33% of the population decline seen at the mainland breeding site Boulder Beach to climate changes. In 2015, the Boulder Beach site had showed a 76% decline in breeding pairs since 1996, demonstrating the vulnerability of mainland yellow-eyed penguin populations (Mattern *et al.*, 2017). Some suggest that the South Island population of yellow-eyed penguins is limited from a further northward expansion by these climate effects (Clark *et al.*, 2015). Further, as climate shifts lead to warmer temperatures on land and at sea in the yellow-eyed penguin's habitat this is likely to put further pressure on the declining populations.

1.2.1.5 Mortality events and disease

The mainland population of yellow-eyed penguins has shown significant overall decline with projected worsening due to climate change (Mattern *et al.*, 2017). The mainland population has been monitored extensively, with a dramatic decline seen in breeding pairs at 9-10 sites along the Otago Peninsula since 1992 (Ellenberg and Mattern, 2012). These intensive nest counts show that in 2011-12 there was estimated to be 452 breeding pairs, while in 2015 the breeding pairs had dropped to just 216 on the South island of New

Zealand (Mattern *et al.*, 2017, BirdLIfe International, 2018). The isolated sub-Antarctic population has received little monitoring by comparison, but recent surveys suggest the population of yellow-eyed penguins occupying the Auckland Islands has remained steady overall despite large annual fluctuations (Muller *et al.,* 2020a). The mainland population is estimated to have declined by 50-79% overall in the last 21 years though (BirdLIfe International, 2018). This decline is attributed to the threats outlined above, and due to episodes of disease in adults and chicks and seasons of mass mortality. The mainland population has undergone multiple mass mortality events, of varying suspected causes. These mass mortality events have not been documented in the sub-Antarctic population, but this may be a result of infrequent monitoring of the isolated population (Muller *et al.* 2020a). The most significant decline in the mainland population occurred in the summer of 1989-1990 where 150 adult birds (estimated population 500) died on the Otago Peninsula (Gill and Darby, 1993). The trigger of this mortality remains unclear, with an unidentified phytotoxin suggested as the possible cause (Gill and Darby, 1993). In other seasons inadequate food supply or decreased prey quality have been suggested as causes of increased adult and chick mortality (van Heezik and Davis, 1990). Between January and March 2013 another epidemic mortality cluster occurred with 67 birds found dead on the Otago Peninsula. Extensive investigation into this mortality event showed consistent histological lesions between birds of pulmonary, hepatic and splenic erythrophagocytosis and haemosiderosis. However, despite exhaustive testing a definitive cause for the mortality event could not be identified, and authors implicated potential exposure to a toxic marine organism (Gartrell *et al.*, 2017).

In addition to the mass mortality events, a number of diseases have contributed to the decline of the mainland yellow-eyed penguin population. Diphtheritic stomatitis was first recognised in yellow-eyed penguin chicks in the 2002-03 season (Alley *et al.*, 2004). Since this initial presentation diphtheritic stomatitis has caused substantial mortalities in chicks in alternate years, from breeding sites across the lower South island and southern offshore islands of New Zealand (Alley *et al.*, 2017). The syndrome is characterised by thick yellow diphtheritic membranes overlying ulceration of the oral cavity grossly. Chicks 1-4 weeks old are affected with emaciation and death in many affected birds (Alley *et al.*, 2004, Alley *et al.*, 2017). The exact causative agent is yet to be identified, but the disease is believed to be caused by primary infection with a poxvirus and then secondary bacterial infection of the oral cavity (Alley *et al.*, 2017). Retrospective analysis of 234 chicks submitted to Wildbase Pathology between 2002 to 2014 from the mainland population showed 113 / 234 chicks were affected (Alley *et al.*, 2017). Furthermore, 11/32 dead chicks were diagnosed with diphtheritic stomatitis from Stewart Island in the season of 2006-07 leading to poor reproductive success in this region (Hill *et al.*, 2010). Diphtheritic stomatitis represents a severe threat to the mainland populations of yellow-eyed penguins as infected chicks frequently succumb to starvation or other complications of the oral infection (Alley *et al.*, 2017).

Finally, haemoparasites have been implicated as diseases of significance in yellow-eyed penguins.

Leucocytozoon has been identified as causes of mortality in wild yellow-eyed penguins, but the significance of these parasites to the population remains unclear. Mortality due to infection with *Leucocytozoon* spp. was identified in 2/14 chicks necropsied from Stewart Island (Hill *et al.*, 2010). Histologically the chicks showed severe, disseminated megaloschizont formation throughout the lungs, liver, spleen, kidneys and other tissues consistent with *Leucocytozoon*osis (Alley *et al.*, 2005, Hill *et al.*, 2010). The significance of *Leucocytozoon* spp. was investigated further via by Argilla *et al.* (2013) through a survey of mainland and sub-Antarctic birds for *Leucocytozoon* spp. by PCR of blood samples. Argilla *et al.* (2013) found a significantly different prevalence of *Leucocytozoon* spp. in 2006/07 between the Enderby Island yellow-eyed penguins (73.7%) and those of Campbell Island (21%) and the South Island (11%). In addition, Argilla *et al.* (2013) identified the *Leucocytozoon* sp. infecting Enderby Island birds to be genetically distinct from the other yellow-eyed penguin populations. This study suggested potentially significant differences in host pathogen dynamics between the sub-populations of the yellow-eyed penguin.

The consequence of *Plasmodium* spp. infection in yellow-eyed penguins has been a controversial area of research. Due to the known susceptibility of penguins to avian malaria, repeated surveys have been performed to investigate these haemoparasites in the yellow-eyed penguin. These studies are discussed in detail later in this review. Avian malaria has been reported to cause clinical disease and mortality in yellow-eyed penguins (Alley, 2001, Hunter *et al.*, 2016) but the prevalence and significance of the pathogen to the population remains unclear. Avian malaria is an emerging disease in New Zealand due to an increasing host range and prevalence (Schoener *et al.*, 2014, Alley and Gartrell, 2019). Therefore, further research into the host-pathogen dynamics of this haemoparasite are critical to understand the threat posed to the vulnerable populations of the endangered yellow-eyed penguin.

1.2.2 Avian Malaria

1.2.2.1 Epidemiology

Avian malaria is a mosquito-borne disease caused by intracellular protozoans of the order Haemosporida, genus *Plasmodium.* There are more than 200 species of *Plasmodium* with a widespread distribution, and the ability to infect almost all avian taxa (Grilo *et al.*, 2016, Rivero and Gandon, 2018). Malaria parasites use mosquitoes (Diptera: Culicidae) as vectors, and so the epidemiology of disease is closely related to the ecology and behaviour of the vectors (LaPointe *et al.*, 2016, Fecchio *et al.*, 2017). Mosquitoes of the genus *Culex, Anopheles, Culiseta, Aedes, Mansonia* and *Aedeomya* are believed to transmit avian malaria (Valkiūnas, 2005, Kimura *et al.*, 2010). The life cycle of *Plasmodium* spp. is complex with the parasites requiring two types of host. The protozoa undergo fertilisation, formation of zygotes and asexual sporogony in invertebrate hosts. In the vertebrate host, asexual merogony and sexual gamogony occur (Atkinson, 2008,

Rivero and Gandon, 2018).

Avian malaria is a markedly seasonal disease driven by the availability of vector populations, that is becoming an emerging threat with climate change (Graczyk *et al.*, 1994, Fortini *et al.*, 2015, Niebuhr *et al.*, 2016, Liao *et al.*, 2017, Fortini *et al.*, 2020). *Plasmodium* spp. are distributed worldwide and are generally associated with low-level chronic infections in species that co-evolve with endemic *Plasmodium* spp. (Atkinson, 2008, Atkinson *et al.*, 2013, Rivero and Gandon, 2018). However, the susceptibility of avian species is markedly different, and avian malaria infections have been responsible for mass mortality events under certain conditions. High morbidity and mortality are reported most commonly in captive birds, due to exposure to novel lineages or immunosuppression, or in wild populations due to the introduction of the parasite to previously naïve hosts (Schoener *et al.*, 2014, Fortini *et al.*, 2015, Paxton *et al.*, 2016). Penguins are known to be highly susceptible to avian malaria infection, resulting in severe and often lethal disease particularly in captive populations (Grilo *et al.*, 2016, Vanstreels *et al.*, 2016). The introduction of *Plasmodium* spp. to Hawaii provides a well-documented example of the potential pathogenicity of avian malaria infection in wild birds. Epizootic *Plasmodium* spp. infection caused the extinction of native avian species in Hawaii and significantly restricted the distribution and abundance of surviving native forest birds (Van Riper III *et al.*, 1986, Fortini *et al.*, 2015, Liao *et al.*, 2017).

Avian malaria is an emerging disease, with the effects of climate change enhancing the spread and incidence of disease. The success of the parasitic infection depends on a number of factors including the presence and abundance of vectors and compatible parasites, the population density of avian hosts and sufficient opportunities for vector feeding (Jones and Shellam, 1999, Medeiros *et al.*, 2015, Ellis *et al.*, 2017, Fecchio *et al.*, 2017). Garamszegl (2011) demonstrated that the prevalence of *Plasmodium* spp. infection in avian hosts is strongly and positively associated with temperature anomalies, with a one degree rise in global temperature having an associated two to three-fold increase in cases of malaria in birds. This increased prevalence of malaria may be explained by warmer temperatures enhancing parasite and vector reproduction, and allowing the spread of vectors into naïve populations and environments (Garamszegl, 2011, Atkinson *et al.*, 2014, Fortini *et al.*, 2020). In addition, anthropogenic influences such as habitat alteration may also be involved in the emergence of vector borne diseases such as avian malaria (Harvell *et al.*, 2002, Hunt *et al.*, 2017). The emerging disease risk of avian malaria coupled with the known susceptibility of penguins to infection makes avian malaria a disease of significance to global penguin populations. For the yellow-eyed penguin populations, the more northern population on the South Island of New Zealand may therefore be expected to be at greater risk of avian malaria than the sub-Antarctic population as the vectors of avian malaria are present in the Otago Peninsula (Tompkins and Gleeson 2006, Ammar *et al.*, 2019, Cane *et al.*, 2020)).

1.2.2.2 Clinical Disease and Pathology

Avian malaria is a disease of the blood and reticuloendothelial system (Atkinson, 2008). The clinical signs and pathology caused by avian malaria infection depends on the strain of parasite and the species of bird infected (Schoener *et al.*, 2014, Rivero and Gandon, 2018). Avian species vary markedly in their susceptibility to avian malaria, and strains are capable of causing mortality in the right conditions (Schoener *et al.*, 2014). There are four stages of infection related to the life cycle of the parasite within the host. The prepatent phase is first and describes the initial stages of merogony of the parasite before entry into the blood stream, lasting two days to several months (Atkinson and van Riper III, 1991, Valkiūnas, 2005). The acute phase occurs second, where the parasites enter the circulation, and rapidly reproduce. Next is the crisis phase, when the parasitaemia peaks causing the greatest pathophysiological stress on the host (Valkiūnas, 2005, LaPointe *et al.*, 2012). The result of infection is determined by the complex interplay between the pathogenicity of the infecting strain and the host's immune response (Videvall *et al.*, 2020). If the bird is capable of surviving the acute and crisis phases of infection, then the infection becomes chronic or latent, and may persist for years or the life of the bird. Infection can remain asymptomatic throughout, with low parasitaemia and chronic infections particularly common in wild birds (Podmokła *et al.*, 2014, Gudex-Cross *et al.*, 2015, Sijbranda *et al.*, 2017a). The pathology and clinical signs caused by avian malaria mirror increases in parasite numbers. The fate of infection varies greatly depending on the strain of *Plasmodium* spp. and the susceptibility of the host to infection.

Avian malaria can cause significant morbidity and mortality in susceptible species, with clinical signs and pathology occurring during the acute and crisis phases of infection. Clinical signs are nonspecific and it is common to find dead birds without preceding clinical signs, particularly in penguins (Vanstreels *et al.*, 2014a, Grilo *et al.*, 2016). The clinical signs seen with acute infection typically include inappetence, anorexia, lethargy, pale mucous membranes, dyspnoea, weakness, weight loss, vomiting and green faeces. In severe and often terminal cases, neurological signs may also be seen including motor incoordination, opisthotonos, convulsions, and paralysis (Atkinson, 2008, Lublin *et al.*, 2018, Taunde *et al.*, 2019). Acute *Plasmodium* spp. infection cause gross pathological lesions including hepatomegaly, splenomegaly, pulmonary oedema and discolouration of the liver and spleen. Malarial pigment accumulates in the macrophages of the liver and spleen causing them to appear dark in colour (Atkinson, 2008, LaPointe *et al.*, 2012). In addition, pericardial effusions, cardiomegaly and nephromegaly may be seen, especially in penguins (Vanstreels *et al.*, 2014a, Hunter *et al.*, 2016). Histopathology allows identification of tissue meronts in endothelial cells, especially in the lungs, brain, spleen, liver and heart. In addition, infiltration of leucocytes into infected tissues is visible with associated haemorrhage (Lublin *et al.*, 2018). Avian *Plasmodium* spp. do not invade hepatocytes though (Valkiūnas, 2005). Pathology is believed to occur as a result of direct haemolysis due to erythrocytic infection, and also vascular occlusion and rupture due to tissue meronts within endothelial cells. These disruptions to the blood and reticuloendothelial systems lead to hypoxia, apoptosis and necrosis of affected tissues and ultimately mortality

due to respiratory insufficiency or circulatory shock (Valkiūnas, 2005, Atkinson, 2008, Vanstreels *et al.*, 2016).

The literature remains unclear on the extent of the subclinical effects of chronic malaria in wild birds. There is evidence that chronic infections in regions where avian malaria is endemic may affect the long-term survival, reproductive success and general fitness of infected hosts (LaPointe *et al.*, 2012, Rivero and Gandon, 2018). Other studies have demonstrated no apparent effect of parasitism on physiological traits, but a possible effect of parasite exposure on migratory behaviour (Kelly *et al.*, 2020). Correlations between avian malaria status and decreased offspring recruitment have been shown (Asghar *et al.*, 2011), as well as apparent fitness effect costs that vary with infection strains (Lachish *et al.*, 2011). Inferences about the fitness effects of chronic avian malaria based on correlational data alone is inherently problematic though due to confounding ecological factors, and the short times scales of the correlational studies (Rivero and Gandon, 2018). Experimental models have presented further evidence for a negative correlation between avian malaria and overall host fitness though (Knowles *et al.*, 2010). In addition, recent long-term studies of great reed warblers, have demonstrated that avian malaria significantly reduces the lifespan of infected hosts through a greater rate of telomere shortening in the cells of infected individuals (Asghar *et al.*, 2015). The long-term implications of chronic avian malaria are an area of ongoing research, with evidence of significant impacts on the lifetime reproductive success and survival of infected hosts.

1.2.2.3 Diagnosis

There is some debate over the best diagnostic method for the detection of avian malaria, as each method has advantages and limitations. There is agreement amongst authors that a combination of methods be used to achieve the greatest accuracy (Zehtindjiev *et al.*, 2012, Schoener *et al.*, 2014, Grilo *et al.*, 2016, Vanstreels *et al.*, 2016). Each method will be discussed briefly below.

Blood smears

Blood smears can be used to examine cells for erythrocytic meronts, and are considered by some authors to be the gold standard for diagnosis of *Plasmodium* spp. *(Grilo et al., 2016).* Thin blood smears are stained with Giemsa and have a higher sensitivity when prepared from capillary blood than venous samples (Mischlinger *et al.*, 2018). Experienced professionals also increase the accuracy of blood smear examination as identifying *Plasmodium* spp. can be both time consuming and difficult (Schoener *et al.*, 2014). The conclusive diagnosis of *Plasmodium* spp. infection through blood smears requires identification of erythrocytic meronts unique to the species of parasite (Valkiūnas, 2005). However, due to the poor sensitivity of smears it is possible to miss low level parasitaemias common in wild birds with latent infection (Miller *et al.*, 2001, Schoener *et al.*, 2014). In addition, acute infections can cause mortality with limited parasitaemia, making blood smears especially unreliable in species such as penguins (Cranfield *et al.*, 1994, Miller *et al.*, 2001). A

negative smear cannot be considered sufficient alone to rule out infection with *Plasmodium* spp. especially when screening for chronic low level parasitaemia (Richard *et al.*, 2002, Fallon *et al.*, 2003). Blood smears remain critical though in detecting co-infections of haemoparasites particularly in wild populations (Zehtindjiev *et al.*, 2012). Confirmation of diagnosis must be performed by combining smear examinations with histopathology and molecular methods to maximise the accuracy of this testing (Zehtindjiev *et al.*, 2012, Vanstreels *et al.*, 2016).

Molecular methods

Molecular tools have a much greater sensitivity than microscopy and are able to detect low levels of parasitaemia in the blood stream. As a result, these tools have been used increasingly as a screening tool for haemosporidian in wild birds (Feldman *et al.*, 1995, Miller *et al.*, 2001, Schoener *et al.*, 2014). RT-PCR (real-time PCR) and nPCR (nested PCR) protocols have been developed to target either the nuclear 18S ribosomal subunit chromosomal gene (18S rRNA), or the mitochondrial cytochrome b gene (cyt-b). There is some debate over the performance and limitations of the methods. Authors agree that PCR methods are more reliable than blood smear analysis for large-scale screening of birds, and that blood smear examination alone may substantially underestimate rates of parasitism (Richard *et al.*, 2002, Fallon *et al.*, 2003, Valkiunas *et al.*, 2008, Friedl and Groscurth, 2012). Although the sensitivity of these methods is much greater than blood smears, false negatives can still occur with inadequate DNA extraction or insufficient concentration of parasite DNA in the sample (Richard *et al.*, 2002). In addition, some species of *Plasmodium* may evade PCR detection, as the performance of the molecular tools varies amongst lineages (Valkiunas *et al.*, 2008). In addition, in samples with co-infections of *Plasmodium* spp.*,* general PCR protocols tend to amplify only the dominant lineage, not necessarily reflecting the pathogenic strain of infected hosts (Zehtindjiev *et al.*, 2012). PCR positive results can also occur even when the infection is abortive for the parasite, due to amplification of DNA from remnants of tissue meronts or sporozoites (Palinauskas *et al.*, 2016). Nonetheless, PCR methods have a significantly higher sensitivity than smears, and so are very useful for screening of wild birds. Microscopy examination can be used to complement population testing via molecular techniques.

RT-PCR protocols offer some advantage over nPCR protocols when screening wild bird populations, with expected low levels of chronic parasitaemia. The RT-PCR protocol (qPCR) developed by Friedl (2012) allows the quantification of the parasite load of infected hosts, compared to nPCR protocols that detect infection status alone. This technique has therefore been used to estimate parasitaemia in wild bird populations surveyed (Zehtindjiev *et al.*, 2012, Sijbranda *et al.*, 2017a). In addition, RT-PCR has been demonstrated to have a higher sensitivity and specificity compared to nPCR, and the highest detection probability in low intensity infections (Ishtiaq *et al.*, 2017, Sijbranda *et al.*, 2017a). The improved sensitivity and ability to quantify pathogen load are advantages of RT-PCR protocols compared to conventional nPCR techniques.

Gross pathology and histopathology

The gross and histopathological lesions associated with *Plasmodium* sp. infection were summarised in detail above. Impression smears can be prepared from the cut surface of the kidney, liver, spleen or lungs to assist in the diagnosis as well (Vanstreels *et al.*, 2014a). In addition, Dinhopl *et al.* (2011) validated an in-situ hybridization technique in penguins, to target a fragment of 18S rRNA, and aid in the detection of *Plasmodium* spp. in paraffin wax-embedded tissues.

ELISA

Graczyk *et al.* (1994) developed an indirect enzyme-linked immunosorbent assay (ELISA) to test penguins for antibodies again *Plasmodium* spp. The assay has been reported as rapid, inexpensive and sensitive and has been used to screen a number of wild penguin populations (Graczyk *et al.*, 1995, McDonald, 2003). However, several authors have questioned the accuracy of the testing given the discrepancies between high seroprevalence and rarely detected parasitaemia (McDonald, 2003, Sturrock and Tompkins, 2007). In addition, there is possible cross-reactivity with *Leucocytozoon* spp. meaning serological results should be interpreted with caution (Vanstreels *et al.*, 2016). Furthermore, positive results do not correlate with parasitaemia and cannot be used to identify active clinical cases, as a positive result can be generated from a single exposure (Grilo *et al.*, 2016). Until detailed studies examine the limitations of serological testing, antibody results should be interpreted with caution.

1.2.2.4 *Plasmodium* in penguins

Avian malaria is one of the most important diseases in captive penguins. Penguins are considered highly susceptible to avian malaria with outbreaks of disease capable of causing mortality in 50-100% of birds within a few weeks (Grilo *et al.*, 2016, Taunde *et al.*, 2019). *Plasmodium* spp. have been detected in thirteen species of penguin in the wild or captivity (Vanstreels 2016). The exact reason for the high susceptibility of penguins remains unclear. It has been suggested that penguins did not co-evolve with *Plasmodium* spp. and therefore, did not develop immunological adaptations to deal with infection (Jones and Shellam, 1999, Valkiūnas, 2005), making them highly susceptible to disease. In addition, due to a lack of co-evolution the parasite would not have been selected for the ability to cause infection without mortality in penguin hosts (Grilo *et al.*, 2016). Avian species which have evolved with malaria are more likely to show resistance to infection or tolerance, where infection does not evoke a strong immune response, one of the key factors in reduction of mortality due to avian malaria (Henschen and Adelman, 2019). Penguins held in captivity in zoos and rehabilitation centres are at an increased risk of infection due to enhanced exposure to vectors, with local avifauna acting as a potential source of infection (Grilo *et al.*, 2016, Sijbranda *et al.*, 2017b, Taunde *et al.*, 2019). Seasonal patterns of infection are observed with the highest mortality occurring in summer months

when vectors are most prevalent (Lublin *et al.*, 2018, Taunde *et al.*, 2019). The key to management of avian malaria in captive penguins is through prevention, as the effectiveness of treatment is significantly reduced once clinical signs are evident. Three approaches are used to minimise the impact of malaria via reduction or exclusion of vectors, drug prophylaxis or allowing natural infection and immunity with regular screening for relapses (Grilo *et al.*, 2016).

Avian malaria is a significant threat to the success of rehabilitation of sick and injured wild penguins. Wild penguins treated in rehabilitation facilities are at risk of acquiring avian malaria due to exposure to high numbers of vectors, but also via recrudescence. Wild penguins stressed due to rehabilitation may develop disease due to recrudescence of sub-patent infections they acquired in the natural environment (Jones and Shellam, 1999, Grilo *et al.*, 2016). A number of studies have investigated the prevalence and impact of *Plasmodium* spp. infection on penguins undergoing rehabilitation. Studies of African penguins admitted to a wildlife rehabilitation facility (The Southern African Foundation for the Conservation of Coastal Birds or SANCCOB) showed that a significant number of penguins were admitted with parasitaemia and that *Plasmodium* spp. infection caused 23% of the mortality seen in rehabilitation penguins over 2 years. In addition, malaria positive penguins remained in captivity for 70% longer than negative penguins (Parsons and Underhill, 2005), and that *Plasmodium* infection status was significantly linked to natural mortality during rehabilitation (Parsons *et al.*, 2018). A further study by Botes *et al.* (2017) suggested that the main source of avian malaria infection of the African penguins in rehabilitation was from infections acquired in captivity rather than via recrudescence. This finding was confirmed with a significant reduction in the prevalence of avian malaria following the introduction of vector control measures, and highlighted the importance of preventative measures in rehabilitation.

An outbreak of avian malaria in 28 Magellanic penguins in rehabilitation in Brazil resulted in an infection rate of 60.7% via PCR screening with an associated mortality of 32.1% (Vanstreels *et al.*, 2014a). Further screening of Magellanic penguins in rehabilitation centres across Brazil by Vanstreels *et al.* (2015) showed an estimated true prevalence of avian malaria of between 6.6-13.5%. This study also demonstrated 44% higher mortality in penguins infected with *Plasmodium* spp. in rehabilitation. In addition to the mortality avian malaria can cause in rehabilitation facilities, it also poses a wider risk to wild penguin populations. Authors have suggested that some penguins may become chronic carriers of *Plasmodium* spp. from rehabilitation and could introduce novel lineages to naïve wild penguin populations (Brossy *et al.*, 1999, Vanstreels *et al.*, 2015, Botes *et al.*, 2017). However, clusters of mortality due to avian malaria in wild penguins have not been recorded to date. Further research into avian malaria dynamics in wild and rehabilitation penguins is critical to understand the potential consequences of rehabilitation efforts.

The exact impact of avian malaria on wild penguin populations is unknown, but the potential for

Plasmodium spp. to cause significant disease in endangered or isolated populations is cause for concern and monitoring (Levin *et al.*, 2009). Avian malaria has the potential to have devastating effects when introduced to naïve and susceptible populations of wildlife. This is demonstrated by the decline and subsequent extinction of endemic birds in Hawaii following the introduction of vectors and *Plasmodium relictum* to the archipelago (Van Riper III *et al.*, 1986, Atkinson and LaPointe, 2009). Due to the high susceptibility of penguins, avian malaria has been identified as a significant threat to the conservation of wild penguins, particularly in tropical and subtropical regions (Brossy *et al.*, 1999, Jones and Shellam, 1999, Tompkins and Gleeson, 2006, Levin *et al.*, 2009, Meile *et al.*, 2013, Vanstreels *et al.*, 2014a, Grilo *et al.*, 2016). Studies have been performed to survey wild penguin populations, but the significance of the disease to the health and fitness of wild penguins remains unclear. Surveys of Antarctic penguin populations have been negative to date (Woods, 2009, Vanstreels *et al.*, 2014b), but there are growing reports of avian malaria in wild penguins of tropical and temperate regions. *Plasmodium* spp. infection has been documented in wild African penguins in South Africa, Northern Rockhopper penguins at Gough Island (Fantham and Porter, 1944), and yellow-eyed penguins, snares and little penguins in New Zealand (Fantham and Porter, 1944, Laird, 1950, Van Rensburg, 2010). *Plasmodium* spp. has recently been detected in Galapagos penguins, after previously negative surveys suggesting the recent introduction of the parasite to the isolated population (Levin *et al.*, 2009, Levin *et al.*, 2013). None of the wild penguins positive for *Plasmodium* spp. showed external evidence of disease with generally low or undetectable levels of parasitaemia on blood smear. However, there is growing evidence of sporadic mortality in wild penguins in New Zealand due to *Plasmodium* spp. infection (Hunter *et al.*, 2016, Sijbranda *et al.*, 2017b). In addition, up to 35% African penguins admitted to rehabilitation in South Africa were positive for *Plasmodium* spp. infection at admission, with authors suggesting the parasite contributed to the debilitation of the infected birds (Parsons and Underhill, 2005). Avian malaria poses a significant potential threat to wild penguin populations, and the impact of infection on wild populations remains unclear. Further research is essential to understand the host-pathogen dynamics, especially in isolated and endangered populations of wildlife such as New Zealand's unique avifauna.

1.2.2.5 Avian malaria in New Zealand

Avian malaria is an emerging disease in New Zealand due to an increasing prevalence and host range (Alley and Gartrell, 2019). Schoener *et al.* (2014) provided a review of the disease stating that 19 lineages of *Plasmodium* spp. have been found so far in 39 different species of birds. The prevalence and distribution of avian malaria across the country is linked to climate dynamics and vector distribution (Tompkins and Gleeson, 2006, Sturrock and Tompkins, 2008, Howe *et al.*, 2012). The rising temperatures as a result of climate change make avian malaria an increasing threat to New Zealand native species. Even modest increases in temperature can enhance vector and parasite replication, and facilitate vector spread into new regions (Holder *et al.*, 1999, Tompkins and Gleeson, 2006, Schoener *et al.*, 2014). Introduced species such as black birds (*Turdus merula*) have been shown to be carriers of at least three strains of *Plasmodium* spp., and are suggested to be reservoirs

of infection for native species (Tompkins and Gleeson, 2006, Schoener *et al.*, 2020). Surveys of native and introduced species have shown a significantly higher prevalence of chronic infections in exotic species, compared to native and endemic birds supporting this conclusion (Howe *et al.*, 2012, Sijbranda *et al.*, 2016, Schoener *et al.*, 2020). In addition, the lower prevalence of infection, coupled with mortality seen in native species suggests New Zealand birds may not have evolved with exposure to lineages of *Plasmodium* spp. leading to less immunocompetence (Howe *et al.*, 2012, Sijbranda *et al.*, 2016). Avian malaria has caused acute infection, illness and mortality in several species of penguins in New Zealand (Hunter *et al.*, 2016, Sijbranda *et al.*, 2017b), as well as hihi (*Notiomystis cincta*) and great spotted kiwi (*Apteryx haastii*) (Howe *et al.*, 2012), saddlebacks (*Philesturnus* sp.) (Tompkins and Gleeson, 2006), and mohua (*Mohoua ochrocephala*) (Alley *et al.*, 2008). Due to an increasing prevalence and host range, and apparent susceptibility of native species to disease, avian malaria poses a threat to the unique biodiversity of New Zealand avifauna.

1.2.2.6 *Plasmodium* in yellow-eyed penguins

The impact of avian malaria on yellow-eyed penguin populations remains unclear, and further research is required to understand host-pathogen dynamics. A number of studies have been performed to investigate if avian malaria contributes to the population fluctuations of the yellow-eyed penguin. *Plasmodium* sp. were first identified in blood smears from wild yellow-eyed penguins from the Fouveaux Strait and Campbell Island by Fantham and Porter (1944) and Laird (1950). Studies since this initial identification have shown contradictory results, with evidence of high seroprevalence of anti-*Plasmodium* antibodies (Graczyk *et al.*, 1995, McDonald, 2003) but no evidence of *Plasmodium* spp. infection with screening via blood smears or PCR (McDonald, 2003, Sturrock and Tompkins, 2007). The discrepancies between these results have been explained by either transient infection in juveniles that were cleared in surviving adults, or due to inaccuracies of the serological tests used (Sturrock and Tompkins, 2007, Vanstreels *et al.*, 2016). Alley (2001) provided the first evidence of mortality due to *Plasmodium* infection in an individual wild juvenile yellow-eyed penguin. Recently Hunter *et al.* (2016) have described an apparent increase in sporadic mortality due to infection by *Plasmodium elongatum* in yellow-eyed penguins, Fiordland crested penguins and an erect crested penguin. These birds were either from the wild or in rehabilitation at the time of mortality, and infection was confirmed via histopathology and PCR analysis. The growing evidence of mortality in yellow-eyed penguins due to *Plasmodium* sp. infection coupled with the contradictory results of previous surveys, highlights a crucial need for further research to understand the impact of this parasite on yellow-eyed penguin populations.

1.2.3 Coccidia

1.2.3.1 Epidemiology

Coccidia are a large and complex group of obligate intracellular parasites in the phylum Apicomplexa. Coccidia infect all classes of vertebrates and many are important pathogens, but this review will focus on

Eimeria spp. that infect wild birds. Classification of coccidia is based on the morphological appearance of the organisms, particularly the final appearance of the sporulated oocyst. Sporulated *Eimeria* oocysts contain 4 sporocysts, each filled with two sporozoites (Fayer, 1980). *Eimeria* have a complex monoxenous life cycle involving both asexual and sexual stages of reproduction in epithelial cells. Most avian species of *Eimeria* develop in intestinal epithelial cells, however extraintestinal infection occurs in some species (Yabsley, 2008). This review will discuss intestinal and renal *Eimeria* as these are the most significant to Sphenisciformes.

Transmission of intestinal *Eimeria* infections is exclusively via ingestion of sporulated oocysts. Unsporulated oocysts are passed in the faeces of avian hosts and must sporulate in the environment before becoming infective. *Eimeria* oocysts are extremely resistant in the environment, and sporulation is driven by environmental conditions (Parker and Jones, 1990, Belli *et al.*, 2006). During sporulation asexual reproduction called sporogony occurs within the resistant oocyst to produce sporocysts and sporozoites (infective stage) from the germ ball (Fayer, 1980, Yabsley, 2008).

Once the sporulated oocyst is ingested it excysts to release the sporocysts which also release the sporozoites. The sporozoites invade intestinal epithelial cells and transform into trophozoites. The trophozoites reproduce asexually within the intestinal epithelial cells to form meronts. These meronts then undergo merogony to produce merozoites (Page and Haddad, 1995, Yabsley, 2008). The infected intestinal epithelial cells are destroyed releasing the merozoites to invade new epithelial cells. The merozoites either undergo repeated rounds of merogony or commence gametogony. The number of cycles of merogony that occur differs among *Eimeria* species (Yabsley, 2008). Merozoites undergo gametogony to form macrogametocytes and microgametocytes. The microgametocytes bud to form many flagellated microgametes, while macrogametocytes mature into a single macrogamete. The microgametes exit the cell to enter cells containing macrogametes where fertilisation occurs with the zygote, developing into an oocyst. The oocyst ruptures the intestinal epithelial cell to be passed in the faeces of the avian host, and commence the life cycle again (Page and Haddad, 1995, Yabsley, 2008).

The life cycle for renal *Eimeria* species is similar to intestinal *Eimeria,* except sporozoites invade and develop in renal tubular epithelial cells rather than intestinal epithelial cells. Once formed, the unsporulated oocysts are passed via the ureters to the cloaca. The oocysts of renal *Eimeria* species are also shed in faeces to sporulate and become infective (Yabsley, 2008). *Eimeria* are generally considered to be very host specific with limited replication and shedding if infection of aberrant hosts occurs (Looszova *et al.*, 2001). Coccidiosis is sporadically reported in wild birds but is principally a disease of captivity and crowding precipitated by stress or concomitant disease (Ladds, 2009, Yabsley, 2008). Overall, little is known about coccidiosis in wild avian hosts, with further research required to understand the significance of coccidial disease in free-living avian populations.

1.2.3.2 Clinical signs and pathology

The clinical effects of enteric infection with *Eimeria* spp. are dependent on the infective dose, species of coccidia and host age and immunity. Coccidiosis is rare in free-ranging birds, and is usually associated with captive rearing, crowding and stress (Ruff and Wilkins, 1987). Coccidiosis is capable of causing severe disease and mortality with ingestion of large numbers of oocysts, particularly in young and naïve animals (Ruff and Wilkins, 1987, Trigg, 1967). As coccidia organisms invade and destroy epithelial cells, the clinical signs observed worsen as the infective dosage increases (Ruff and Wilkins, 1987, Yabsley, 2008). Low level infections destroy only a small number of intestinal epithelial cells, which are rapidly replaced. Birds with low level infections may show no evidence of clinical disease. However, moderate to high intensity infections can destroy large numbers of intestinal epithelial cells. This leads to inappetance, dehydration, decreased intestinal absorption of fluid and nutrients and also intestinal haemorrhage (Yabsley, 2008). Therefore, the clinical signs observed in cases of clinical coccidiosis include diarrhoea (with blood and mucus), lethargy, listlessness, inappetance, emaciation, decreased weight gains, ruffled feathers, ataxia, and decreased egg production and death (Trigg, 1967, Hunt and Ogrady, 1976, Ruff and Wilkins, 1987). The course of clinical disease is also dependent on the host's immunity, the species infecting the host and the age of the bird (Ruff and Wilkins, 1987, Yabsley, 2008).

Pathologic changes with enteric *Eimeria* infection vary widely based on the parasite species, host and severity of infection. The gross pathological lesions caused by intestinal coccidiosis include splenomegaly, depletion of fat reserves, ballooning of intestines, intestinal hyperaemia, petechial to multifocal intestinal haemorrhage, and red-brown intestinal contents (Yabsley, 2008, Ladds, 2009). Histological examination shows the development stages of coccidia readily within intestinal epithelial cells. In cases of coccidiosis there is focal necrosis or attenuation of intestinal crypt epithelium with accompanying heterophilic or mononuclear infiltration. In addition, there may be distension of the intestinal crypts and glands with cellular debris and sometimes overgrowth of coliform bacteria (Yabsley, 2008, Ladds, 2009,).

Most coccidial organisms infecting avian hosts occur within the intestinal tract, with some coccidia developing in extra-intestinal sites. The most common site for extra-intestinal development of some coccidia species is in renal tubular epithelial cells (Yabsley, 2008). Renal coccidioisis has been noted mostly as an incidental finding in wild seabirds of Australia and New Zealand with limited or no pathology (Alley *et al.*, 2004, Ladds, 2009). Gross pathological lesions associated with severe renal coccidiosis have included pale enlarged kidneys, sometimes with multifocal to coalescing white spots in the kidneys and dilation of the ureters and cloaca (Munday *et al.*, 1971, Harrigan, 1992). Histological pathology reported with renal coccidiosis varies greatly from the occasional oocyst in the lumen of ducts and renal epithelium to distension of ureters with oocysts and severe nephritis (Munday *et al.*, 1971, Obendorf and McColl, 1980, Alley *et al.*, 2004).

1.2.3.3 Diagnosis

The diagnosis of avian coccidiosis is performed through the identification of oocysts in faeces coupled with clinical signs of disease or via the examination of tissues histologically at necropsy (Yabsley, 2008). The detection of coccidia oocysts in faecal samples relies on the separation of oocysts from faecal material using solutions of sucrose, ZnSO4 or saturated NaCl that allow oocysts to float in the faecal suspension (Ryley *et al.*, 1976). The final structure of the sporulated oocysts can be used to differentiate the coccidia genera. *Eimeria* spp. contain four sporocysts, each with two sporozoites while *Isospora* spp. have two sporocysts, each with four sporozoites (Belli *et al.*, 2006). Tissue stages are often logistically difficult or impossible to obtain in wildlife research, and so detailed descriptions of the morphological characteristics of oocysts in faeces can be used to describe new species of Eimeriidae (Duszynski and Wilber, 1997). In addition, a variety of quantitative techniques are used to examine a known aliquot of faecal sample and estimate the infection as the number of oocysts per gram of faeces (Vadlejch *et al.*, 2013, Cringoli *et al.*, 2017). The traditional microscopic methods of species identification are also now complemented by molecular diagnostic techniques. PCR assays have been used to differentiate *Eimeria* spp. in poultry, and with further development have become useful in studies of wild avian populations (Chapman, 2014).

1.2.3.4 Coccidiosis and wild birds

Overt disease due to coccidia infection is rarely seen in avian hosts under normal conditions in the wild (Alley and Gartrell, 2019). Wild birds are commonly exposed to only low numbers of oocysts, allowing hosts to develop immunity, and making clinical disease rare (Yabsley, 2008, Merino, 2010). However, under certain conditions, outbreaks of disease and mortality can occur. Captive rearing, crowding, malnutrition, pre-existing diseases and stress of wild birds can lead to the development of coccidiosis and significant pathology and mortality (Ruff and Wilkins, 1987, Yabsley, 2008, Alley and Gartrell, 2019). For example, significant mortality has been observed in juvenile captive reared kiwi exposed to high numbers of oocysts prior to the development of immunity (Morgan *et al.*, 2014). In addition, high mortality in wild juvenile little penguins has been documented, due to suspected overwhelming oocyst infection in non-immune juveniles, and inclement weather (Harrigan, 1992). Therefore, as parasitism in wild birds is linked to immunosuppression of the hosts, coccidia are potentially useful ecological biomarkers to detect challenges to the host-pathogen relationship (Berto and Lopes, 2020). Although disease and mortality are rare in wild birds, coccidiosis may also exert population level effects. For example the disease may reduce the reproductive success of wild birds by decreasing clutch sizes and hatchling success and survival (Ruff *et al.*, 1984, Ruff *et al.*, 1988, Watson, 2013, Bertram *et al.*, 2015). Outbreaks of coccidiosis occur sporadically in wild birds when conditions cause exposure to high numbers of oocysts in hosts with compromised or no immunity, making them useful potential biomarkers.

1.2.3.5 Coccidiosis in wild penguins (including renal coccidiosis)

The prevalence and impacts of coccidian infections in wild penguins remain unclear. A number of studies have reported no or low burdens of coccidia in wild penguins, but some authors have also suggested coccidiosis to be a disease of potential significance, particularly in isolated populations (Golemansky, 2008, 2011). Studies in wild rockhopper penguins, gentoo penguins, emperor penguins and chinstrap penguins found no evidence of coccidia in any sample (Karesh *et al.*, 1999, Vidal *et al.*, 2012, González-Acuña *et al.*, 2013, Kleinertz *et al.*, 2014). However, Golemansky (2011) suggested that coccidia could have a significant clinical effect on free-living penguin populations after surveying the three *Pygoscelis* species of penguins using faecal samples collected over 6 years. Golemansky (2011) reported 35% of the 360 samples were positive for coccidia, with a high burden (80-220 oocysts/field) reported in 20/126 (15.9%) positive samples. Another factor limiting the understanding of coccidia in penguins, is frequent reports of the recovery of coccidial oocysts that could not be sporulated (Obendorf and McColl, 1980, Carrera-Játiva *et al.*, 2014, Golemansky, 2016). Surveys of wild Galapagos penguins in 2014 (Carrera-Játiva *et al.*) found 14.4% prevalence of coccidia in faecal samples, but all samples remained unsporulated and so could not be examined in morphological detail. The studies of *Pygoscelis* penguin species (Golemansky, 2016) has shown mixed infections of *Eimeria* and *Isospora* in all three species of penguin, and evidence of 3 separate species of coccidia. However, only the species *Eimeria pygosceli* (Golemansky, 2003) has been identified due to failure of the remaining oocysts to sporulate. The prevalence and significance of coccidia infections in wild penguins remains unclear, with varying prevalence results and difficulty sporulating oocysts. Further research is required.

Renal and intestinal coccidiosis has been documented in wild little penguins of Australia and New Zealand. Infections are more common in juvenile birds, and most penguins have only mild and localised lesions associated with renal and intestinal coccidian infection (Obendorf and McColl, 1980, Alley, 2002, Cannell *et al.*, 2016). There is evidence that renal and intestinal coccidiosis can lead to severe pathology in wild little penguins though, especially when infection is associated with starvation and exhaustion (Obendorf and McColl, 1980, Harrigan, 1992, Van Rensburg, 2010). Obendorf and McColl (1980) necropsied little penguins following a mortality event in Victoria in 1980 and found 38.7% of the 31 kidneys examined had renal coccidiosis histologically. The histological lesions were mild and focal, apart from in 2 positive birds, where severe and generalised lesions were seen including blockage of renal collecting ducts, intrarenal ureteritis, and chronic interstitial nephritis. Van Rensburg (2010) showed a lower prevalence of renal coccidiosis in New Zealand little penguins but found five out of six birds had severe pathology histologically. Harrigan (1992) demonstrated severe intestinal and renal coccidiosis in juvenile Australian little penguins, with nearly 100% prevalence, while few adult penguins had evidence of parasitism. In summary, while coccidia infection in juvenile penguins appears to be common, coccidiosis resulting in severe pathology in juvenile and adult penguins is infrequent, but is exacerbated when combined with environmental stressors and decreasing food

supply. Further study is required to understand endemic coccidia prevalence in penguin populations, to better understand host-pathogen dynamics.

1.2.3.6 Coccidia and the yellow-eyed penguin

Little is known about coccidiosis in yellow-eyed penguins. Only two reports have been published describing coccidial oocysts in the faeces of yellow-eyed penguins. The first description of coccidia in yellow-eyed penguins was reported by Ranum and Wharton in 1996. The authors found 35% of the 20 faecal samples examined were positive for coccidial oocysts. The sporulated oocysts were identified as an *Eimeria* species based on the morphology. A low number of oocysts was reported in the samples, with counts ranging from 2-265 oocysts/gram faeces. The highest burden of oocysts was seen in a single sample with 8402 unsporulated oocysts/gram faeces. Ranum and Wharton (1996) concluded that the low oocyst counts observed suggested that coccidial protozoa were unlikely to cause significant disease in yellow-eyed penguin populations, unless the penguins experienced concomitant stress or disease. The authors called for further research to understand host-pathogen dynamics, and the relationship between parasite load and the success of birds in the wild.

The second description of coccidial oocysts in yellow-eyed penguin faecal samples was by McDonald in 2003. The author reported 18.5% of the 65 faecal samples collected were positive for coccidia. Oocyst counts seen in this study were also low with only three birds having oocyst counts greater than 100 oocysts/gram of faeces. These oocysts were again identified as *Eimeria* based on sporulated morphology, and most oocysts were sporulated at the time of examination. However, due to the destruction and degradation of the sporulated oocysts examined, the authors questioned whether the coccidia seen originated from the fish prey of the birds and were unable to describe the morphology of an intact sporulated oocyst in detail. In addition to these studies of coccidia in faeces, Alley *et al.* (2004) also provides a report of sporadic renal coccidiosis in yellow-eyed penguins at necropsy. Between 1995-2003, 125 birds were necropsied from the South Island of New Zealand. During this study, Alley *et al.* (2004) identified occasional renal coccidiosis as an incidental finding in young birds. The oocysts were seen in the renal collecting tubules histologically and were commonly associated with little inflammatory reaction. Alley *et al.* (2004) proposed that coccidial parasitism may be less important in the yellow-eyed penguin compared to colony forming penguins due to their solitary breeding habitat. Little is known about coccidiosis in yellow-eyed penguins with few reports of coccidial oocysts from birds of the mainland population, and no reports of coccidia from the sub-Antarctic birds. Further research is required to understand host-pathogen dynamics of coccidia in the yellow-eyed penguin, particularly to examine if the varying habitat conditions of the two sub-populations of this endangered species has an impact on pathogen load.

1.3 Research Aims and Thesis Structure

The yellow-eyed penguin is a unique and endangered species facing a number of growing threats to the persistence of the species (BirdLIfe International, 2018). The population is comprised of two geographically and genetically distinct population centres, occupying different habitat ranges and faced with different environmental stressors (Boessenkool *et al.*, 2009a, 2009b, 2010, Seddon *et al.*, 2013). Little is known about differences in population trends between the two strongholds, but there is evidence to suggest pathogen prevalence and mortality rates vary between the two populations (Argilla *et al.*, 2013, Argilla, 2015).

Disease surveillance of the geographically distinct yellow-eyed penguin populations is critical to inform future conservation efforts and predictions of population responses to ongoing climate shifts. *Plasmodium* and *Eimeria* are both parasites that have been detected in yellow-eyed penguins previously, but there is a paucity of research describing the prevalence and pathogen load of these parasites within the wild populations.

The aims of this study was to explore possible differences in host-pathogen dynamics between the two population centres of the yellow-eyed penguin, and to identify what implications changing disease dynamics may have for the future management of the species. This was achieved by investigating the following hypotheses:

1) There will be a difference in the prevalence of avian malaria between the two major population centres of yellow-eyed penguins
2) There will be a higher prevalence of avian malaria in birds in rehabilitation compared to wild populations of yellow-eyed penguins
3) The prevalence of *Eimeria* will vary between the population centres of the yellow-eyed penguin
4) The *Eimeria* species infecting yellow-eyed penguins is a novel species that has not been formally described

This thesis is organised into four chapters, comprising two data chapters. The first chapter provides a review of yellow-eyed penguin ecology and population dynamics, the pathophysiology of avian malaria and the impacts of infection on wild and captive penguins, and the epidemiology and pathology of coccidian infections in wild birds and penguins. The second chapter explores the prevalence and pathogen load of *Plasmodium* sp. infection within free-living yellow-eyed penguins (2015-2017) and those in rehabilitation (2014). The third chapter investigates the prevalence of *Eimeria* spp. infection in the mainland and sub-Antarctic populations of wild yellow-eyed penguins (2015-2017), and formally describes a novel *Eimeria* sp. The fourth chapter comprises a general discussion, which provides a conclusion for the thesis and explores the conservation implications of the host-parasite systems investigated in this study. The data chapters presented in this thesis are written in a format allowing for subsequent publication.

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CHAPTER TWO

Investigation into the prevalence and pathogen load of *Plasmodium* in yellow-eyed penguins

CHAPTER TWO – INVESTIGATION INTO THE PREVALENCE AND PATHOGEN LOAD OF *PLASMODIUM* IN YELLOW-EYED PENGUINS

2.1 Introduction

Avian malaria is a widespread mosquito borne disease that is an emerging disease threat to the unique native avifauna of New Zealand. Avian malaria is caused by protozoans of the genus *Plasmodium* and is showing an increased prevalence and host range in New Zealand (Alley and Gartrell, 2019). The parasites generally cause low-grade chronic infections in avian hosts that co-evolved with *Plasmodium* spp. in their habitat (Valkiūnas, 2005, Atkinson, 2009). However, severe and lethal disease can result from infection, particularly in naïve individuals or susceptible species. This has been famously demonstrated by the role of avian malaria in the decline and extinction of Hawaiian avifauna, and the ongoing avifauna distribution restrictions due to avian malaria infection patterns (Van Riper III *et al.*, 1986, Atkinson and LaPointe, 2009, Fortini *et al.*, 2015).

In New Zealand, introduced species of birds are suspected to act as reservoirs for avian malaria, showing a higher tolerance to the circulating stages of the parasite (Sijbranda *et al.*, 2016, 2017b). These introduced species of birds are believed to act as sources for infection in native and endangered species of New Zealand avifauna (Tompkins and Gleeson, 2006). Acute and lethal infections with high levels of parasitaemia have been documented in a number of native New Zealand passerine species, penguin species and great spotted kiwi (*Apteryx haastii)* (Tompkins and Gleeson, 2006, Alley *et al.*, 2008, Howe *et al.*, 2012, Hunter *et al.*, 2016, Sijbranda *et al.*, 2016, 2017b). The true impact of chronic avian malaria infections on native New Zealand birds remains unclear though, and some authors have suggested that *Plasmodium* sp. infections may be contributing to the ongoing decline of native avian populations (Tompkins and Jacob Hoff 2011, Niebuhr 2016) as a secondary factor to major causes of extinction such as habitat loss and degradation and introduced mammalian predators (Garcia and Di Marco, 2020).

Internationally, penguins are considered highly susceptible to avian malaria, and the parasite is one of the most important causes of morbidity and mortality in captive populations. The exhibition of penguins in open air enclosures in zoos, and admission of birds into rehabilitation facilities may increase exposure to malarial vectors, and increase the risk of infection with local *Plasmodium* strains maintained in less susceptible avifauna (Sijbranda *et al.*, 2017b, Lublin *et al.*, 2018, Taunde *et al.*, 2019). In addition, the stress of

rehabilitation may cause recrudescence of naturally acquired chronic *Plasmodium* spp. infection (Parsons and Underhill, 2005). Infection prevalence in zoos and rehabilitation centres can range from 6-43% (Vanstreels *et al.*, 2015, Grilo *et al.*, 2016), and up to 100% during outbreaks (Taunde *et al.*, 2019). High mortality has been reported during seasonal outbreaks of disease in captive populations of penguins, with the highest mortality in summer months during peak vector densities (Lublin *et al.*, 2018, Taunde *et al.*, 2019). However, *Plasmodium* spp. have been rarely detected from wild penguins, and the impact of the parasite on the health and fitness on wild penguin populations remains unclear.

Although avian malaria outbreaks in captive penguins are well documented, the impact of the parasite on the health and fitness of wild penguin populations remains unclear. Diagnosis of *Plasmodium* spp. infection has been made in six species of wild penguin from temperate and tropical regions, via microscopic examination of blood smears alone (Fantham and Porter, 1944, Laird, 1950, Alley, 2001, Thiart, 2005). A single case (1/154) of *Plasmodium relictum* infection in a little penguin (*Eudyptula minor)* in New Zealand in 2006 was identified via blood smear examination and molecular sequencing (Van Rensburg, 2010). More recently, nested PCR was used to demonstrate an estimated prevalence of *Plasmodium* spp. infection in Galapagos penguins (*Spheniscus mendiculus)* of 5% (19/362), with authors suggesting infection in this species may be abortive (Levin *et al.*, 2009, 2013). All of the penguins in the aforementioned studies had no external signs of disease, with a generally low level parasitaemia. However, some authors have suggested a possible role of *Plasmodium* spp. infection in the debilitation of African penguins (*Spheniscus demersus)* admitted for rehabilitation at the Western Cape in South Africa (Grilo *et al.*, 2016). Parsons and Underhill (2005) observed that 35% of penguins were positive within 5 weeks of admission, compared to the estimated prevalence of < 1% in the wild African penguins of that region (Fantham and Porter, 1944, Brossy *et al.*, 1999, Thiart, 2005). In addition, Sijbranda *et al.* (2017b) recently documented sporadic cases of mortality in little penguins (*Eudyptula minor)* in New Zealand, both in the wild (2/274) and in captivity (2/21) and suggested the combined use of blood smears and real-time PCR methods to detect both acute and low level chronic infections in penguins that may be missed with other techniques.

Avian malaria has been a demonstrated cause of mortality in wild yellow-eyed penguins (*Megadyptes antipodes*), but the dynamics of infection in the wild birds remain unclear (Alley, 2001, Hunter *et al.*, 2016). The first reports of *Plasmodium* spp. infection was recorded by Fantham and Porter (1944) and Laird (1950) via blood smear examination. Since this initial description of the parasite, the results of testing in this species have been contradictory. Graczyk *et al.* (1995) demonstrated 100% seroprevalence (n = 5) of antibodies to avian malaria via ELISA, and suggested the possible role of avian malaria in the decline of the mainland yellow-eyed penguin population of New Zealand. Alley (2001) then described the first case of clinical disease and mortality in a juvenile wild yellow-eyed penguin from the Otago Peninsula, with infection confirmed via histopathological examination. McDonald (2003) demonstrated a consistently high seroprevalence in wild

yellow-eyed penguins, with 97.5% positive with ELISA (n = 78/80), but failed to identify any evidence of parasitism via blood smear examination (n=0/83). Sturrock and Tompkins (2007) then screened 143 wild penguins from mainland New Zealand for avian malaria via PCR, and all were negative.

Authors have suggested a number of reasons for the discrepancies between these study results including, inaccuracies of the serological tests used (McDonald, 2003, Sturrock and Tompkins, 2007, Vanstreels *et al.*, 2016), poor sensitivity of the PCR tests used (Palmer *et al.*, 2013) or due to transient infections in juveniles that were not adequately sampled (Sturrock and Tompkins, 2007). Most recently, Hunter *et al.* (2016) has reported further cases of sporadic mortality in yellow-eyed penguins (n = 3), as well as a Fiordland crested penguin and an erect crested penguin. These birds were either in rehabilitation, or in the wild of the South Island of New Zealand at the time of death. Infection was confirmed via histopathology and PCR, with sequencing identifying *Plasmodium elongatum* in all five cases. This evidence of mortality in wild yellow-eyed penguins coupled with the contradictory results of previous surveys highlights the need for further research.

The yellow-eyed penguin is endemic to New Zealand, and is one of the most endangered penguin species in the world (BirdLIfe International, 2018). Genetic analysis has shown that there are two genetically and geographically distinct populations of yellow-eyed penguins – the mainland New Zealand South Island population (including Stewart island and surrounding islands) and the sub-Antarctic population (Boessenkool *et al.*, 2009a, 2009b, 2010). These distinct population centres are estimated to comprise a total of approximately 1700 breeding pairs, with at least 60% of the population in the sub-Antarctic (Moore, 1992, McKinlay, 2001, Moore, 2001, Seddon *et al.*, 2013, Muller *et al.,* 2020). The two populations live in vastly different habitats, with the mainland birds occupying the topmost extreme of the home range and facing different marine and terrestrial threats than the sub-Antarctic birds (Boessenkool *et al.*, 2009a, Boessenkool *et al.*, 2009b, Boessenkool *et al.*, 2010, Seddon *et al.*, 2013, Argilla, 2015, Robertson, 2017). The mainland population has been monitored extensively, showing overall significant decline and also episodic mass mortality events (Gill and Darby, 1993, Ellenberg and Mattern, 2012, Gartrell *et al.*, 2017, Mattern *et al.*, 2017). The population dynamics of the sub-Antarctic birds is less well understood due to their isolated habitat, but the population appears to be stable overall despite large interannual variation (Muller *et al.* 2020). Recent research has suggested significant potential differences in disease dynamics between the two population centres though, with a significantly higher prevalence of the haemoparasite *Leucocytozoon* occurring in the sub-Antarctic population (Argilla *et al.*, 2013).

The known susceptibility of penguins to *Plasmodium* infection, coupled with the evidence of mortality in yellow-eyed penguins suggest the potential for avian malaria to be a pathogen of significance to this endangered population. Previous surveys for avian malaria in this species have been contradictory. In addition,

the two distinct populations of yellow-eyed penguins are challenged by different stressors, and have shown differences in disease dynamics of another haemoparasite *Leucocytozoon* (Argilla *et al.*, 2013). Therefore, this study aimed to investigate the hypotheses that there would be differences in avian malaria prevalence between the two major population centres of yellow-eyed penguins, and secondly that there would be a higher prevalence of avian malaria in young birds in rehabilitation compared to wild populations.

2.2 Materials and method

2.2.1 Study sites

Yellow-eyed penguins were sampled from wild sites to represent the mainland and sub-Antarctic populations. Birds were sampled from the wild of south eastern Otago (45.8667° S, 170.6667° E) and the Catlins Coast (46.4636° S, 169.1972° E) to represent the mainland population of the South Island of New Zealand. Birds were also sampled from Rocky Ramp at Enderby Island, part of the Auckland Island archipelago, to represent the sub-Antarctic population of birds (50°29'45"S 166°17'44"E) (**Figure 2.1**).

Yellow-eyed penguins were also sampled opportunistically as part of general health monitoring while in hospital and at rehabilitation facilities. These facilities included the wildlife hospitals Wildbase in Palmerston North, and the Dunedin Wildlife Hospital, and rehabilitation facilities along the Otago Peninsula (**Figure 2.1**). All birds housed in these facilities were originally from wild sites at the Catlins or Otago Peninsula.

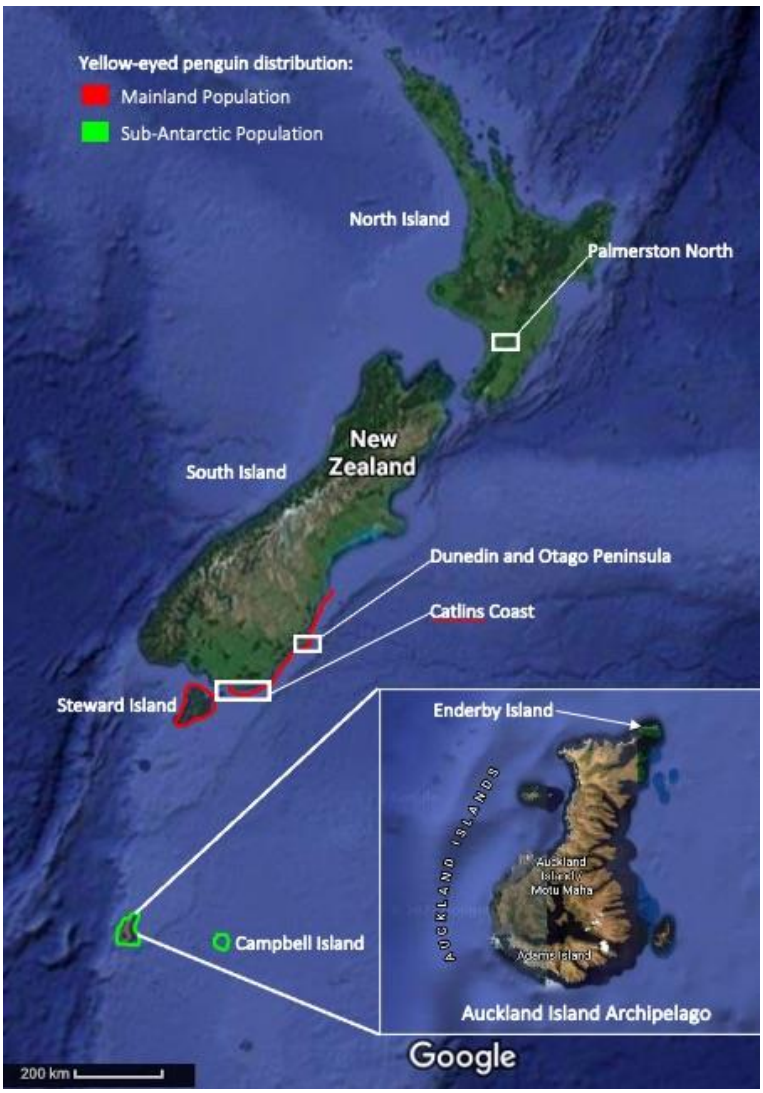

Figure 2. 1 Population distribution of yellow-eyed penguins (*Megadyptes antipodes*) and sampling sites.

2.2.2 Collection of blood samples and handling

Adult wild yellow-eyed penguins were opportunistically selected on Enderby island (n = 65) during the breeding season between November 2015 – January 2016. Blood samples were collected from each individual to screen for the presence of *Plasmodium*. Each bird was identified by a subcutaneous transponder, and so no birds were sampled twice.

Adult wild birds were also randomly selected and sampled from the Otago Peninsula and Catlins coast (n = 73) between Feb 2017 – June 2017. In addition, 5 wild chicks (defined by having down feathers present) were sampled opportunistically during this time as part of medical care. Again, all birds were identified by a subcutaneous transponder, and so no birds were sampled twice.

Yellow-eyed penguins were opportunistically sampled as part of health monitoring during medical

care and rehabilitation between April – June 2014 (n = 44). This sample group consisted of 8 adults, 10 juveniles, 21 chicks, and 5 birds of unknown age, and were in human care for between 1-57 days prior to sampling. In addition, a further 11 birds were opportunistically sampled in hospital or rehabilitation between January – March 2017 (n = 6 adults, n = 2 chicks, and n = 3 juveniles). Age classes of penguins were defined by the following physical characteristics, with chicks having down feathers present, juveniles were characterised by pale grey irises and lacking the pale yellow band on the dorsal head, while adults had the full adult plumage with distinctive band of yellow feathers around the eyes and caudal head, and a bright yellow iris (Seddon 2013).

All wild birds captured on Enderby Island and on the Otago Peninsula/Catlins coast were given a physical examination, and weighed at capture. Morphometric measurements were taken to allow sexing of birds via the combined crown and beak length, and the foot length, and were confirmed via the database of information for mainland birds where possible. A body condition score was subjectively recorded for each bird using a scale of 1-9 (where 1 is emaciated and 9 is obese) by assessing pectoral muscle mass and subcutaneous fat reserves by palpation. Each bird was also scanned for a microchip, and had a passive integrated transponder placed subcutaneously if no microchip was already present. Where possible the same aforementioned information was gathered for each bird sampled opportunistically in rehabilitation or hospital care.

Blood samples of volumes between 0.5-2 mL were drawn from either the medial metatarsal vein, or the brachial vein of each bird. Four drops of blood were placed directly onto FTA cards, or filter paper, and then four fresh blood smears were prepared per bird on glass slides. The remainder of the blood sample was then placed in lithium heparin blood containers for storage. The blood smears were air-dried and stored in a sealed, dry container prior to fixing with 100% methanol.

The blood smears of wild birds positive for *Plasmodium* via molecular testing were stained with 10% Giemsa solution for 45 minutes (Atkinson, 2009) for microscopic examination. The entire smear was initially screened for haemoparasites using a microscope at a low magnification (400x) for 5-20 mins. Then at least 100 fields were studied closely at a high magnification (x1000) for approximately 30 minutes. No blood smears were available for examination from positive birds in rehabilitation/hospital.

2.2.3 Molecular studies

Nested PCR was used to screen the samples collected from rehabilitation birds in 2014. From this point on, RT-PCR was used to screen all wild penguin samples for *Plasmodium* spp. infection, based on recent

work showing RT-PCR protocols have a higher sensitivity and specificity for detecting low level parasitaemia (Sijbranda *et al.*, 2017a). In addition, samples from wild birds that tested positive via RT-PCR were also analysed using the nested PCR method to allow sequencing of amplified products.

DNA was extracted overnight from blood samples on FTA cards or filter paper using the DNeasy Blood and Tissue Kit (Qiagen, Valencia CA, USA), following the manufacturer's instructions for nucleated whole blood. Samples of extracted DNA were stored in a -20°C freezer until they were used for molecular analysis. The NanoDrop 2000 (Thermo-Fisher, Wilmington, USA) was used to measure the concentration and purity of each sample before molecular analysis.

Nested PCR

Samples were screened for the presence of the cytochrome b gene of *Plasmodium* spp. using a nested polymerase chain reaction (nPCR) based on the protocol by Hellgren *et al.*, (2004). The primers used for the reaction were NF1/NR3 and then F/R2 to amplify a 480 bp fragment of the mitochondrial cytochrome b gene from avian *Plasmodium* species. A known *Plasmodium* positive sample was used for the positive control, and nuclease free water was included in the reaction as the negative control. The final PCR products, after the two cycles of amplification were complete, were run on 1% agarose gel (Invitrogen, Carlsbad, CA, USA) containing ethidium bromide for 45 minutes at 100 V.

Positive amplified products from the nPCR reactions were purified using a PureLink PCR purification kit (Invitrogen, Auckland, New Zealand). These purified products were then analysed to confirm the genomic sequence, using automatic dye-terminator cycle sequencing with the BigDye Terminator Version 3.1 Ready Analyser (Applied Biosystems Inc., Forster City, CA, USA). This sequence was compared with published sequences in GenBank, to identify the causative *Plasmodium* species.

Real-Time PCR

All wild birds were screened using RT-PCR instead of nPCR to maximise the specificity and sensitivity of testing samples with expected low parasitaemia (Fallon *et al.*, 2003, Ishtiaq *et al.*, 2017b, Sijbranda *et al.*, 2017a). To screen the samples for *Plasmodium* sp. DNA, an 85 bp product of the LSU-rRNA gene was amplified using the primers Plasmo474for and Plasmo558rev by Friedl and Groscurth (2012), and the reaction by Sijbranda (2015). The following modifications were made to the aforementioned reaction. Each 20 µl RT-PCR reaction comprised 10 µl of 2x Perfecta SYBR Green Fastmix (Quanta biosciences, Gaithersburg, USA), 0.25 µM of the primers Plasmo474for and Plasmo558rev and 2 µl of sample DNA. Each sample was measured using the NanoDrop 2000 prior to analysis, with DNA concentration in samples ranging from 1.2 ng/µl to 15.6 ng/µl.

Samples with higher concentration were diluted for the reaction so that all samples had a DNA concentration between 1.2 ng/μl to 7.8 ng/μl. The RT-PCR initial steps included 2 minutes at 50°C and 10 minutes at 95°C, followed then by 45 cycling rounds of 95°C for 15 seconds and then 60°C for 30 seconds. Next the pre-melt conditioning was performed at 95°C for 15 seconds, and then a 0.3 degree per second incremental melt curve over a 60-95°C temperature range was made with a 5 second hold after each temperature change. This reaction was performed on the micPCR version 1.8.0 system. Samples were assigned as positive with a melt curve of approximately 78°C, and R^2 values of >99% (Figure 2.2).

As the LSU-rRNA gene is a highly conserved sequence, the above reaction was tested for cross-reactivity between *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. This was critical as mixed infections were anticipated due to the previously documented high prevalence of *Leucocytozoon* in yellow-eyed penguins (Hill *et al.*, 2010, Argilla *et al.*, 2013). The test run using known positive samples of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* showed no amplification of the *Haemoproteus* or *Leucocytozoon* samples. All samples that showed amplification of product consistent with the *Plasmodium* LSU-rRNA gene via the above RT-PCR were also analysed by nested PCR. All samples were negative using nested PCR, despite showing repeated amplification via RT-PCR three times, and therefore could not be sequenced.

Standard curves and RT-PCR for quantification

Standard curves were then made to validate the positive results achieved via RT-PCR screening, and to quantify the amount of *Plasmodium* parasites in each blood sample. To allow this calculation, standard curves were made using known quantities of clones of the 585 bp fragment of the LSU-rRNA gene from a positive blood sample identified as *Plasmodium LINN1*. These vector DNA copies were prepared via the protocol by Sijbranda (2015), and Sijbranda *et al.* (2017a), using the forward and reverse primers L1 and L2 described by Tan *et al.* (1997). The resulting plasmid DNA was analysed using the NanoDrop 2000 (Thermo-Fisher, Wilmington, USA) to confirm the purity of the vector DNA and to quantify the resulting concentration. The purified vector DNA was then diluted using Tris-EDTA (AE) buffer to make a solution of concentration 1 ng/μl. This 1 ng/μl solution was then used to make seven ten-fold dilutions with AE buffer ranging from 1 ng/μl to $1x10^{-7}$ ng/μl. The dilution of 1 ng/μl corresponds to approximately 2.6×10^8 copies of the target sequence/μl (Friedl and Groscurth, 2012). Each of these standard dilutions was stored at 4°C, and used within 12 hours of preparation.

The samples identified as positive via the above RT-PCR method described, were then run with all

seven dilutions above to create standard curves and allow quantification of the *Plasmodium* spp. DNA in the samples and standards. Each qPCR run had Cq-values between 7.9 and 35.0 thermo-cycles using a threshold of between 0.020 – 0.167 (**Figure 2.3**). As the concentration of DNA in the samples was low, 9μl of sample was used for the reactions with 11μl of the mastermix described above in the RT-PCR protocol for a total reaction volume of 20μl. The standard dilutions of plasmid were run with 1μl volume of each dilution and 19μl of mastermix, for a total reaction volume of 20μl. All standard curves were highly reproducible and had R^2-values of 99% (**Figure 2.4**). The standard curves were then used to calculate the concentration of *Plasmodium* DNA in each test sample, using the linear equations generated from the curves (**Figure 2.5**). To convert the calculated concentration of *Plasmodium* DNA in each sample to the number of parasites in the sample, the assumption by Wilson *et al.* (1996) was used. This assumption states that each *Plasmodium* parasite contains a single copy of the LSU-rRNA gene, and therefore the amount of copies of the target sequence amplified in each sample correlates directly to the amount of *Plasmodium* parasite in that sample.

The parasite load was then calculated for all positive samples to reflect the number of parasites per 10,000 of avian blood cells, using knowledge of the genome size of penguins by Tiersch and Wachtel (1991). The average genome size of penguins is 3.26 pg (Tiersch and Wachtel, 1991), and so each ng of total DNA in the unknown samples in a reaction contains approximately 306 blood cells. Therefore, the total amount of DNA in each reaction in ng was multiplied by 306 cells to calculate the number of avian blood cells in the reaction. The parasite load was finally recorded as the number of parasites per 10,000 avian blood cells.

2.2.4 Statistical analysis

The apparent prevalence of *Plasmodium* and 95% confidence interval was calculated using the Epitools software following the Wilson binomial approximation (Brown *et al.*, 2001, Sergeant, 2018). The true prevalence of *Plasmodium* and 95% confidence interval was also calculated using the Epitools software following the Blaker binomial approximation (Sergeant, 2018). To perform the Blaker calculation the sensitivity and specificity of the tests used was estimated based on the results reported in Sijbranda (2015). The estimates used for the calculation are as follows: RT-PCR sensitivity 98%, and specificity 96.7%, and nPCR sensitivity 80.9%, and specificity 85.4%.

Chi-square tests (Preacher, 2001) were used to analyse differences in the prevalence of *Plasmodium* between the sample groups of penguins. In categories where the frequencies were below five, Fisher's exact tests were used instead (Preacher, 2001). The significance of the time in rehabilitation relative to the infection status of the yellow-eyed penguins was also analysed by an ANOVA test. Results are presented as mean +/- 1 standard error (±) unless otherwise stated.

2.3 Results

2.3.1 Infection prevalence based on qPCR in wild birds

Blood samples of 65 wild yellow-eyed penguins from Enderby Island and 72 wild penguins from the South Island of New Zealand, were tested by qPCR during this study (Table 2.1). Samples were considered positive for the presence of *Plasmodium*-specific DNA if the qPCR results revealed dissociation temperatures within the range of 78.0-80.3°C to account for *Plasmodium* spp. variation. The qPCR showed an apparent prevalence for adult yellow-eyed penguins on Enderby Island of 0% (95% CI 0-5.58%), with all 65 birds negative on qPCR. The apparent prevalence for adult yellow-eyed penguins sampled on the South Island of New Zealand was 6.85% (95% CI 2.96-15.05%), with 5/73 adults testing positive. In addition, 1/5 chicks sampled in the wild on the South Island of New Zealand opportunistically were also positive on qPCR for *Plasmodium*-specific DNA. There was good evidence (p = 0.045) for a difference in the prevalence between the Enderby Island and South Island populations.

Of the six wild yellow-eyed penguins from the South Island of New Zealand that tested positive, 5/6 were adult birds, and 1/6 was a chick. The chick was of unknown sex, and 4/5 adult birds were male. All three Catlins sites where birds were sampled had at least one positive bird (Long Point, Penguin Bay and Nugget Point), and one of the three sites (Otapahi beach) sampled from the Otago Peninsula had a positive bird.

All blood smears were negative on examination.

Table 2. 1 The estimated true prevalence of *Plasmodium* spp. infection based on real-time PCR (qPCR) results for wild birds and nested PCR (nPCR) results for birds sampled in rehabilitation, including 95% confidence intervals (CI).

	True Prevalence (Blaker CL)	**True Prevalence 95% CI**	**Apparent Prevalence (Wilson CL)**	**Apparent Prevalence 95% CI**
Enderby Island Wild Adults *Screened via qPCR* **(n = 65)**	0	0 – 0.0241	0	0 – 0.0558
South Island Wild Adults 2017 *Screened via qPCR* **(n = 73)**	0.0375	0 – 0.1241	0.0685	0.0296 – 0.1505

South Island Wild Chicks 2017 *Screened via qPCR* **(n = 5)**	0.1763	0 – 0.6246	0.2	0.0362 – 0.6245
Total Wild South Island (Adults and Chicks) 2017 *Screened via qPCR* **(n = 78)**	0.0464	0.0029 – 0.1318	0.0769	0.0357 – 0.1578
Total Rehab birds (Chicks, Juveniles, Adults and birds of unknown age) 2014 *Screened via nPCR* **(n = 44)**	0.7739	0.5511 – 0.9581	0.6591	0.5114 – 0.7812
Rehab Chicks 2014 *Screened via nPCR* **(n = 21)**	0.7135	0.3964 – 0.9751	0.619	0.4088 – 0.7925
Rehab Juveniles 2014 *Screened via nPCR* **(n = 10)**	0.8356	0.3782 - 1	0.7	0.3968 – 0.8922
Rehab Adults 2014 *Screened via nPCR* **(n = 8)**	1	0.5778 - 1	0.875	0.5291 – 0.9776
Rehab Birds of Unknown Age 2014 *Screened via nPCR* **(n = 5)**	0.3831	0 – 0.9401	0.4	0.1176 – 0.7693

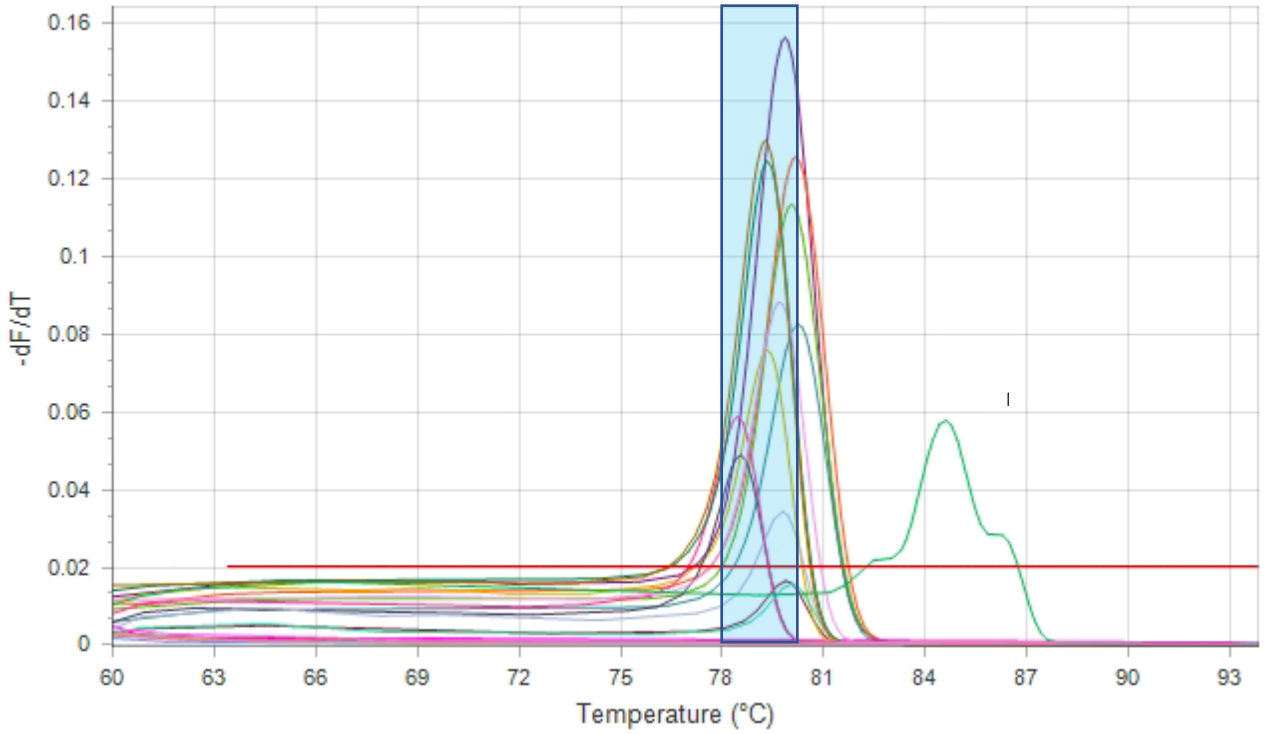

Figure 2. 2 Meltcurves for samples positive for *Plasmodium* specific DNA, and a sample negative for *Plasmodium* specific DNA, but showing the presence of non-specific DNA amplicons. The blue shading shows the range of dissociation temperatures for *Plasmodium* spp.

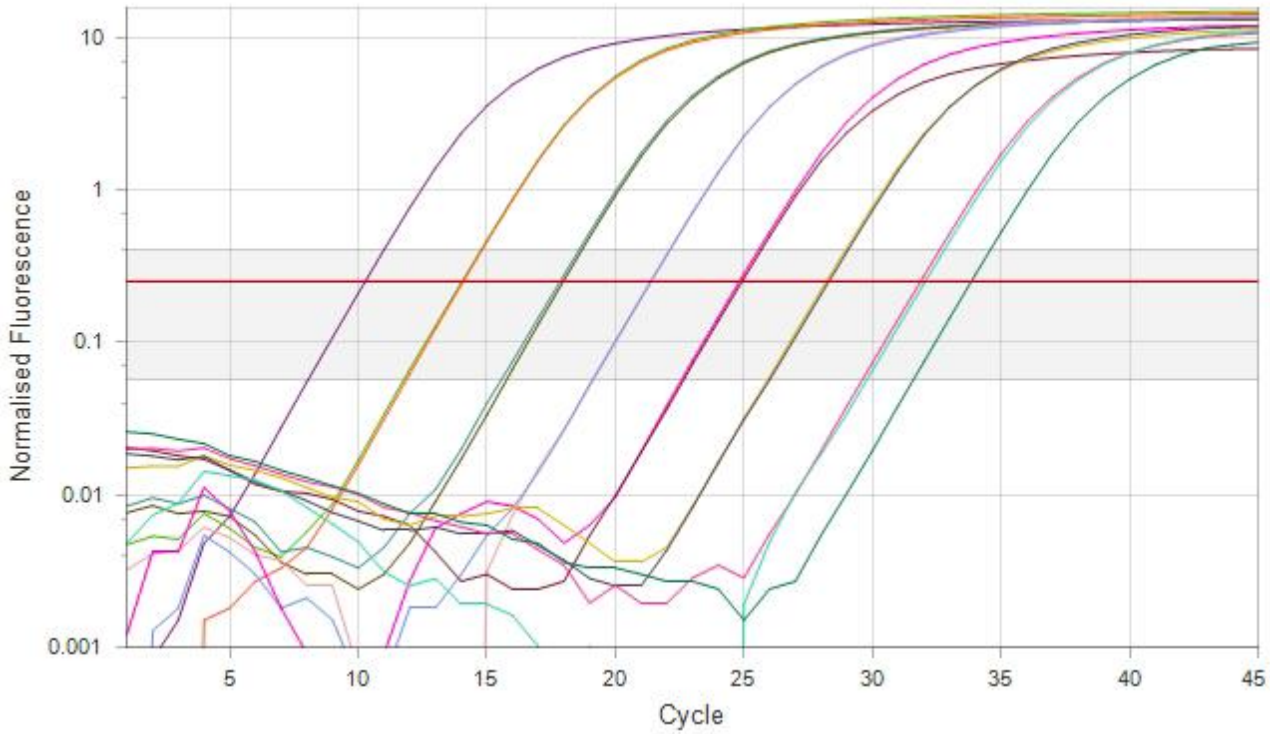

Figure 2. 3 Real-time PCR amplification curves of 7 standard samples with a dilution factor of 10 with a starting quantity of 2.60 x 10^8 copies of the qPCR target DNA sequence (Friedl and Groscurth, 2012). Nuclease free water was used as a negative control.

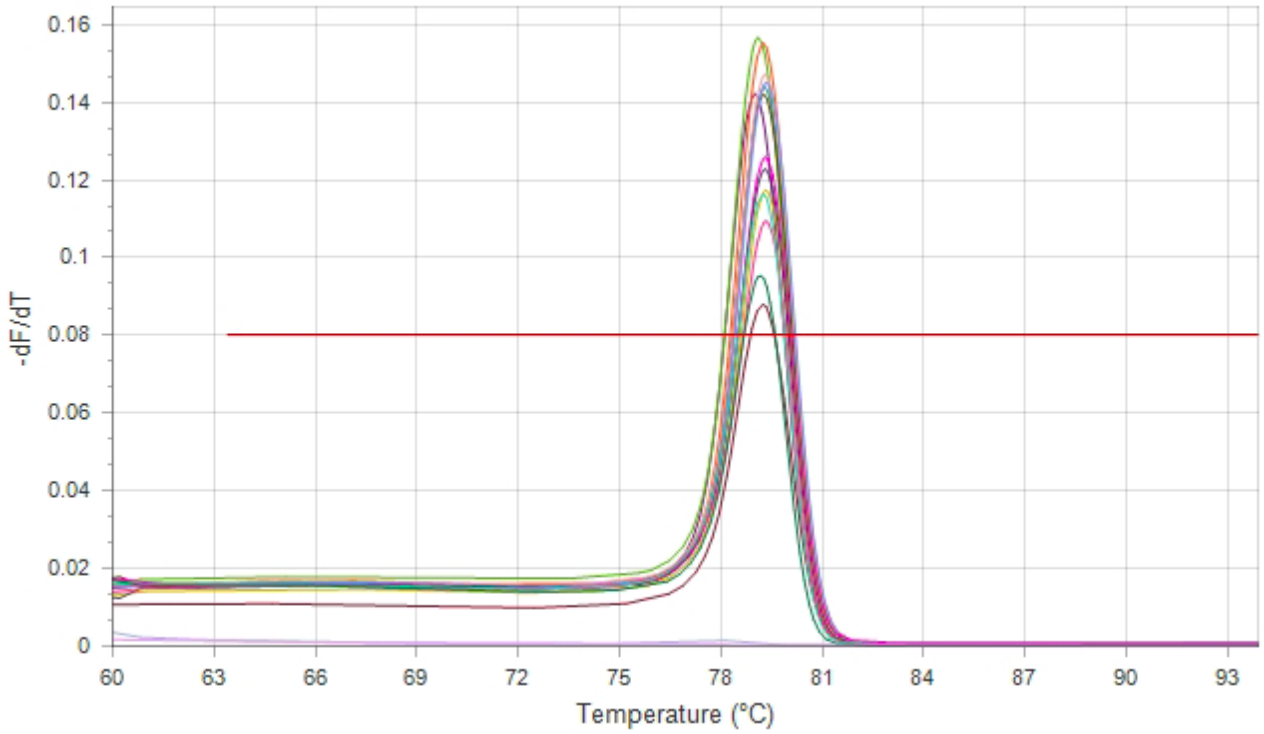

Figure 2. 4 Meltcurves for samples positive for *Plasmodium* specific DNA, and standard samples for quantification of pathogen load. Nuclease free water was used as a negative control.

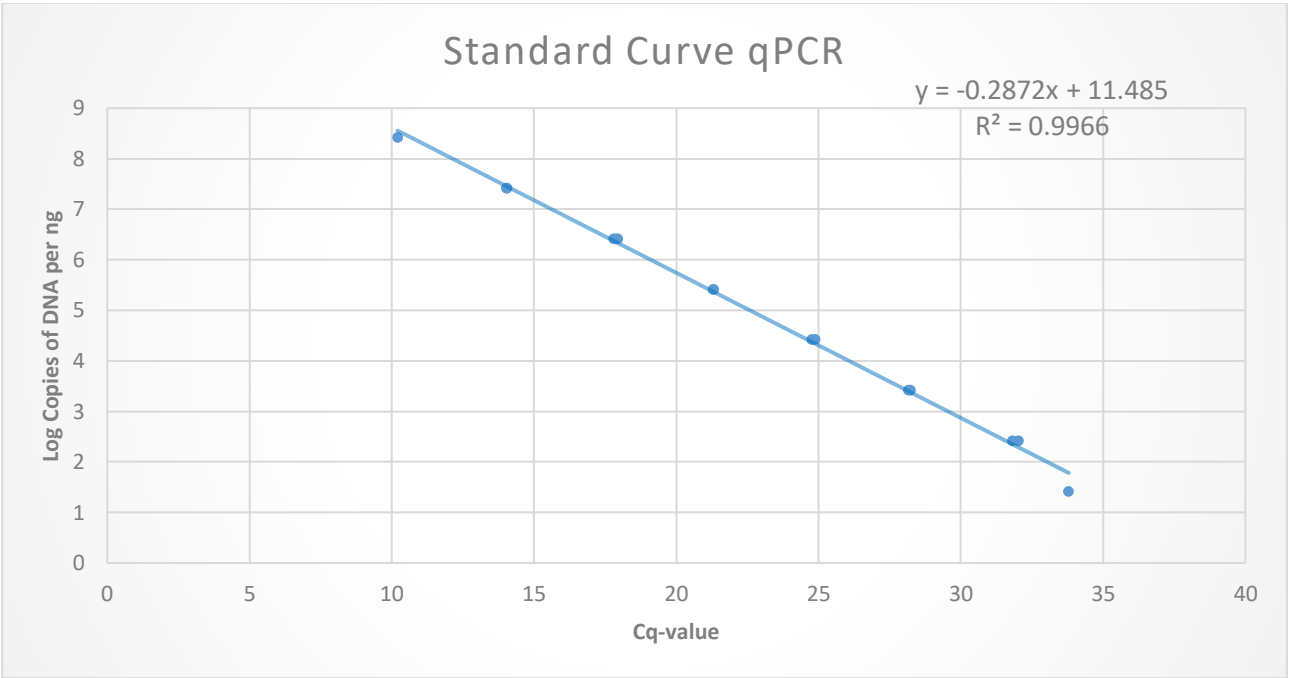

Figure 2. 5 Standard curve of 7 standard samples run in duplicate with a dilution factor of 10 and a starting quantity of 2.60 x 10^8 copies of the qPCR target DNA sequence (Friedl and Groscurth, 2012). Nuclease free water was used as a negative control.

2.3.2 Infection intensity in wild birds

The infection intensity (parasite load) in the 6 wild yellow-eyed penguin qPCR samples ranged from

42-222 *Plasmodium* DNA copies per 10,000 avian blood cells, with a mean pathogen load of 122 *Plasmodium* DNA copies/10,000 avian cells (± 29 *Plasmodium* DNA copies/10,000 avian cells) (Table 2.2). All 6 samples were repeatedly positive when tested via qPCR but were negative on repeated analysis of the samples by nPCR. The lower detection limit of the nPCR protocol used has been reported as likely 30 copies of *Plasmodium* DNA per 10,000 avian cells by Sijbranda *et al.* (2017a), suggesting the pathogen load of all 6 samples should have been within the detection limit of the nPCR protocol.

Table 2. 2 Infection intensity and history for wild yellow-eyed penguins from the South Island of New Zealand identified as positive on qPCR, with parasite load defined as the number of *Plasmodium* DNA copies per 10,000 avian cells

	Location Sampled	History of time in rehabilitation prior to sampling	Gender	Age	Weight *(kg)*	Body condition score	Parasite load
Wild South Island Positive Bird 1	Long Point (Catlins)	No	Male	Adult	6.3	5/9	222
Wild South Island Positive Bird 2	Otapahi Beach (Otago Peninsula)	Yes	Male	Adult	5.7	5/9	85
Wild South Island Positive Bird 3	Penguin Bay (Catlins)	No	Unknown	Chick	3.1	2/9	195
Wild South Island Positive Bird 4	Nugget Point (Catlins)	No	Female	Adult	5.1	4/9	73
Wild South Island Positive Bird 5	Nugget Point (Catlins)	Yes	Male	Adult	5.1	4/9	115
Wild South Island Positive Bird 6	Penguin Bay (Catlins)	No	Male	Adult	5.9	5/9	42

2.3.3 Infection prevalence based on nPCR in rehabilitation birds

Blood samples were collected from 44 yellow-eyed penguins during rehabilitation, including 21 chicks, 10 juveniles, 8 adult birds and 5 birds of unknown age. Overall, 29/44 birds sampled in rehabilitation tested positive for the presence of *Plasmodium* DNA using nested PCR (Table 2.1). The apparent prevalence of

Plasmodium spp. in birds in rehabilitation sampled overall was 65.91% (95% CI 51.14 – 78.12%). The *Plasmodium* spp. prevalence for chicks in rehabilitation was 61.90% (13/21; 95% CI 40.88 – 79.25%), for juveniles in rehabilitation was 70.00% (7/10; 95% CI 39.68 – 89.22%), for adults in rehabilitation was 87.5% (7/8; 95% CI 52.91 – 97.76%) and for birds in rehabilitation of unknown age was 40% (2/5; 95% CI 11.76 – 76.93%) (Table 2.1). There was no evidence ($p = 0.677$) for any difference in prevalence of avian malaria between the age cohorts.

There was very strong evidence ($p < 0.001$) for a higher prevalence of avian malaria infection in adult birds in rehabilitation (87.5%) compared to the wild adult population of the South Island (6.8%). There was no significant difference ($p = 0.0912$) between the prevalence of avian malaria in chicks in rehabilitation (61.9%) compared to the small number of wild chicks sampled (20%).

DNA sequencing was successful for 12 out of 30 *Plasmodium*-positive yellow-eyed penguin blood samples from birds in rehabilitation. For the remaining 18 positive blood samples the PCR product was too weak to sequence successfully. For all 12 of the successfully sequenced samples the nucleotide sequences of the amplified DNA product showed > 99% similarity with known sequences from GenBank as concluded by NCBI BLAST. *Plasmodium* sp. Lineage LINN1 (GenBank GQ471953) was found in four yellow-eyed penguins in rehabilitation (3 chicks and 1 juvenile). *Plasmodium relictum* lineage SGS1 (GenBank AF495571) was found in one yellow-eyed penguin chick in rehabilitation. *Plasmodium elongatum* lineage GRW06 (GenBank DQ368381) was found in 7 yellow-eyed penguins in rehabilitation (3 chicks, 1 adult, 1 juvenile and 2 unknown age).

2.3.4 Relationship between time in rehabilitation and *Plasmodium* status

The time in rehabilitation at sampling, for testing for *Plasmodium* spp. infection via nPCR, was recorded for 34 of the 44 yellow-eyed penguins sampled in 2014. There was weak evidence ($F = 3.391$, $df = 1,32$, $p = 0.075$) for a difference between the mean number of days in rehabilitation at the time of sampling between the positive and negative birds. The mean time in rehabilitation at sampling for yellow-eyed penguins that tested positive for *Plasmodium* sp. via nPCR was 17.7 days (± 14.2 days). Three birds tested positive for *Plasmodium* spp. after being in rehabilitation for 2 days or less. The mean time in rehabilitation at sampling for yellow-eyed penguins that tested negative for *Plasmodium* spp. on nPCR was 28.9 days (± 20.5 days).

2.4 Discussion

This study aimed to investigate the hypotheses that there are differences in avian malaria prevalence between the two major population centres of yellow eyed penguins, and secondly that there would be a higher prevalence of avian malaria in birds in rehabilitation compared to wild populations. The results showed good

evidence for differences in the prevalence of *Plasmodium* spp. between the wild birds of Enderby Island and the wild birds of the South Island of New Zealand, with a higher prevalence in the South Island birds. In addition, there was also very strong evidence for a higher prevalence of *Plasmodium* sp. In birds in rehabilitation compared to wild populations. The observed patterns of *Plasmodium* sp. prevalence may be explained by variation in the infection dynamics at the vector, host and pathogen level between the study populations (Niebuhr et al., 2016).

The wild populations of yellow-eyed penguins sampled for this study are genetically and geographically isolated, occupying vastly different home ranges ((Boessenkool *et al.*, 2009b, 2010). Therefore, it is possible that the observed difference in *Plasmodium* spp. prevalence between the two wild populations reflect differences in the host-pathogen dynamics. The prevalence of *Plasmodium* spp. is vastly influenced by environmental factors such as the climatic conditions, and the distribution and habitat use patterns of the parasite and host (LaPointe *et al.*, 2010, Szöllősi *et al.*, 2011, Svensson-Coelho *et al.*, 2013, Gonzalez-Quevedo *et al.*, 2014, Ellis *et al.*, 2017). In addition, the transmission efficiency of the arthropod vectors, virulence of the *Plasmodium* spp. and the susceptibility and tolerance of the avian hosts all drive the prevalence of infection (Westerdahl *et al.*, 2012, Fecchio *et al.*, 2013, Svensson-Coelho *et al.*, 2013, Gonzalez-Quevedo *et al.*, 2014).

Culex pervigilans is a known vector of *Plasmodium* spp., is distributed widely across New Zealand, and has been identified at both wild population sites (Belkin, 1968, McDonald, 2003). Although this vector may be present at both sites, the relative effect of differences in vector density must be considered. The interplay between *Plasmodium* prevalence, host traits, environmental conditions and vector abundance is still being explored and presents conflict within the literature. Some studies present evidence that the prevalence of haemoparasites are linked to increasing vector density (Wood *et al.*, 2007, Svensson-Coelho *et al.*, 2013). Furthermore, Medeiros *et al.* (2015) demonstrated a positive association between *Plasmodium* prevalence and vector biting rates. Fecchio *et al.* (2017) then identified no relationship between vector abundance and *Plasmodium* prevalence in southeastern Amazonia, suggesting vector utilisation rather than abundance to be a key driver of avian malaria infection. However, Ishtiaq *et al.* (2017a) recently showed host abundance positively influenced *Plasmodium* prevalence while mosquito abundance showed a negative association. Ellis *et al.* (2017) also demonstrated a link between host abundance and the prevalence of *Plasmodium* infection. Alternatively, studies in Hawaii have suggested ongoing links between increasing vector density and the prevalence of avian malaria (Atkinson *et al.*, 2014, LaPointe *et al.*, 2016, Paxton *et al.*, 2016, Fortini *et al.*, 2020). Differences in vector density and utilisation between the two study populations offers one possible explanation for the differences in apparent prevalence of *Plasmodium* spp.in this study, but further research is required to confirm this.

The role of climate and seasonal temperature differences must also be considered in relation to the differences in the observed prevalence of *Plasmodium* spp. infection between the wild populations studied. The successful incubation of *Plasmodium* spp. within the mosquito host is inhibited outside a temperature threshold of between 15-30 °C (LaPointe *et al.*, 2010). Therefore, it is possible that the apparent absence of *Plasmodium* spp. infection in the yellow-eyed penguins of Enderby Island may reflect climatic conditions that are prohibiting the successful development of the *Plasmodium* spp. within the arthropod host. In addition, the lower environmental temperatures of Enderby Island may also result in decreased vector survival and feeding activity, preventing the transmission and maintenance of the *Plasmodium* spp. life cycle (Benning *et al.*, 2002, LaPointe *et al.*, 2010, Schoener *et al.*, 2014). Regardless of the underlying mechanism, this result confirms my hypothesis that there is a difference in avian malaria prevalence between the two major populations of yellow eyed penguins. This may also result in differing patterns of mortality between the two population. However further study would be needed to confirm this possibility.

This study offers the first report of a PCR positive result for *Plasmodium* spp. in wild yellow-eyed penguins. Previous work to investigate the disease dynamics of *Plasmodium* spp. in the wild birds have yielded contradictory results, with a high apparent seroprevalence of antimalarial antibodies but consistently negative PCR screening results (Graczyk *et al.*, 1995, McDonald, 2003, Sturrock and Tompkins, 2007). My study reports an apparent prevalence of *Plasmodium* spp. of 6.85% in the wild yellow-eyed penguins of the South Island sampled. This study used LSU-rRNA qPCR to analyse wild samples instead of cytochrome b nPCR as has been used in previous studies (Sturrock and Tompkins, 2007). The LSU-rRNA qPCR has been shown to have a higher sensitivity to detect low-level parasitaemia compared to the cytochrome b nPCR, and therefore results in fewer false negatives (Sijbranda *et al.*, 2017b). The samples were run in duplicate to confirm repeatedly the positive results, but could unfortunately not be sequenced due to the high conservation of the ribosomal segment of RNA. Therefore, I suggest that these positive results via LSU-rRNA may reflect true positive infections with *Plasmodium* spp. that are below the detection limit of cytochrome b gene nPCR, or that the infections are positive but abortive. Palinauskas *et al.* (2016) showed that positive PCR amplification can result from the DNA of parasite syncytia, which are abortive stages of *Plasmodium* infection within the vertebrate host.

This study showed a significantly higher prevalence of *Plasmodium* spp. infection in yellow-eyed penguins in rehabilitation compared to free-living wild populations sampled. The source of infection in these birds in rehabilitation could be newly acquired disease in hospital, or chronic infections from the wild that

have undergone recrudescence during hospitalisation (Parsons and Underhill, 2005, Botes *et al.*, 2017). The prepatent period of infection is 3-12 days (Valkiūnas, 2005). Three birds tested positive for *Plasmodium* spp. Infection in this study after having been in rehabilitation for only 2 days prior to testing positive, suggesting these 3 birds were infected in the wild. Internationally, reports of *Plasmodium* spp. infection in penguins in rehabilitation have suggested that locally acquired infections are the main source of infection rather than recrudescence (Parsons and Underhill, 2005, Botes *et al.*, 2017). Given the significant difference in the apparent prevalence of *Plasmodium* spp. infection between wild yellow-eyed penguins and those in rehabilitation it appears likely that a significant number of birds were infected during rehabilitation. The stress of illness and hospitalisation coupled with exposure to increased density of vectors are likely drivers of newly acquired *Plasmodium* spp. infections in the penguins in rehabilitation (Vanstreels *et al.*, 2014, 2015, Botes *et al.*, 2017).

Plasmodium spp. has the potential to have a significant impact on the success of rehabilitation efforts of yellow-eyed penguins in New Zealand. Three different strains of *Plasmodium* were identified via sequencing in samples from yellow-eyed penguins in rehabilitation. This variation in the pathogen strains present suggest the increased prevalence of malaria infection in rehabilitation is not due to strain virulence, but instead a combination of host and environmental factors as discussed above. All three strains are commonly maintained in free-living passerine populations in New Zealand (Sijbranda *et al.*, 2016) suggesting possible spill-over of infection into the birds during the rehabilitation process. The three lineages identified in this study have shown variable pathogenicity in the native birds of New Zealand. Lineage LINN1 has been a demonstrated cause of mortality in great spotted kiwis, but otherwise has only been reported in native species in extremely low numbers (Sijbranda *et al.*, 2016). Lineage GRW06 has a wide host range in New Zealand with generally low pathogenicity, but has caused sporadic deaths in wild birds (Howe *et al.*, 2012, Niebuhr *et al.*, 2016, Sijbranda *et al.*, 2016). Lineage SGS1 appears to show a variable severity of disease in birds in New Zealand, with infections causing low level parasitaemia in some species and acute mortality in others (Howe *et al.*, 2012). The infection of yellow-eyed penguins in rehabilitation with *Plasmodium* spp. has the potential to cause direct mortality in affected birds, as well as significantly prolong hospitalisation periods, as has been seen in international rehabilitation facilities (Parsons and Underhill, 2005, Vanstreels *et al.*, 2014). In addition, authors have suggested that penguins infected with *Plasmodium* spp. in rehabilitation could become chronic carriers of the disease, and introduce novel lineages of *Plasmodium* spp. into wild penguin populations with significant morbidity and mortality as a result (Brossy *et al.*, 1999, Vanstreels *et al.*, 2015, Botes *et al.*, 2017). Unfortunately, none of the positive samples from wild yellow-eyed penguins were able to be sequenced to compare with lineages noted in rehabilitation birds. However, the significantly higher prevalence of *Plasmodium* spp. in rehabilitation penguins in this study demonstrates the importance of mosquito preventative measures in rehabilitation facilities. Vector exclusion measures increase the success of

rehabilitation through preventing exposing the penguins to local *Plasmodium* spp. and decreases the risk of introducing novel lineages into the wild population of penguins when rehabilitated birds are released (Botes *et al.*, 2017).

This study provides the first detection of *Plasmodium* spp. infection in wild yellow-penguins. The pathogen load of the penguins varied from 42-222 *Plasmodium* DNA copies per 10,000 avian blood cells. The pathogen load can give possible clues to the host pathogen relationship between wild yellow-eyed penguins and *Plasmodium* spp. The level of parasitaemia observed is a product of the combined effects of the phase of avian malaria infection at the time of sampling, the immune response of the host and the virulence of the infecting *Plasmodium* strain (Westerdahl *et al.*, 2012, Videvall *et al.*, 2020). Levels of parasitaemia vary between the four stages of infection of avian malaria, with peak parasitaemia occurring in the acute and crisis phases of infection, while chronic infections are characterised by typically low levels of circulating haemoparasites (Valkiūnas, 2005). The immune-alleles of avian hosts can produce either higher susceptibility to infection, or can result in qualitative or quantitative resistance to *Plasmodium* infection, thereby influencing circulating levels of parasite (Westerdahl *et al.*, 2012). Over time host-parasite relationships can co-evolve towards a moderated immune response and higher tolerance to the parasite, allowing hosts to carry parasites without the development of severe pathology (Atkinson *et al.*, 2013, Henschen and Adelman, 2019). However, the virulence of the infecting *Plasmodium* spp. also drives levels of parasitaemia, and the hosts response with marked differences demonstrated between lineages (Videvall *et al.*, 2020). These results offer a platform for further work to investigate possible links between pathogen load and infection dynamics within the yellow-eyed penguin host.

Penguins are generally considered to be highly susceptible to disease due to *Plasmodium* sp. infection (Grilo *et al.*, 2016). The prevalence of *Plasmodium* spp. infection in wild yellow-eyed penguins is lower than report rates of infection in endemic and other native New Zealand birds screened by Sijbranda *et al.* (2017a) using the same real-time polymerase chain reaction protocol. Sijbranda *et al.* (2017a) reported an infection prevalence of 21% in endemic species and 11% in other native species screened, compared to the apparent prevalence of 6.9% in yellow-eyed penguins on the South Island and 0% in the birds of Enderby Island. The pathogen load for all positive yellow-eyed penguins was lower than the introduced species (2245 DNA copies per 10,000 avian blood cells) surveyed in Sijbranda *et al.* (2017a) but greater than the native and endemic species surveyed (2.8 and 31.5 DNA copies per 10,000 avian red cells). The lower prevalence and pathogen load of *Plasmodium* spp. infection in yellow-eyed penguins seen in this study compared to the levels reported in introduced species (Sijbranda *et al.*, 2017a), may indicate a higher pathogenicity of infection in yellow-eyed penguins with few penguins surviving infection to test positive in sampling. However, the sample size is too

small to make any true estimations of the pathogenesis of infection. Alternatively, the higher parasite load in the yellow-eyed penguins of this study compared to native and endemic species surveyed by Sijbranda *et al.* (2017a) could indicate that yellow-eyed penguins have a higher tolerance to infection. That is, they are able to maintain higher levels of circulating pathogens, without mounting an immune response that would lead to disease and possible mortality (Henschen and Adelman, 2019). However, the higher parasite load in yellow-eyed penguins could also reflect a decreased ability to lower parasite numbers compared to the other native and endemic species surveyed. Further research is required to understand the host-pathogen dynamics of *Plasmodium* spp. infection in wild yellow-eyed penguins, with this study offering the first description of wild pathogen loads of infection.

Although this study offers new insights into understanding the host-pathogen dynamics of *Plasmodium* spp. infection in yellow-eyed penguins, there are a number of limitations that must be noted. The major limitation of this study is that the penguins were sampled in different seasons from each population. Therefore, different environmental conditions during the survey period could play a significant role in the prevalence of *Plasmodium* spp. across the years of the study. In addition, due to missing clinical health data in the rehabilitation birds sampled, inferences about the impact of infection on the health of the birds could not be made.

Further research is required to understand the host-pathogen dynamics of *Plasmodium* spp. infection on the endangered population of yellow-eyed penguins. The implications of infection remain unclear based on this study and so further work is required. Internationally there Is evidence of the negative impacts of chronic *Plasmodium* spp. infection in wild avian populations (Asghar *et al.*, 2011, Asghar *et al.*, 2015), and there has been growing evidence of mortality in the yellow-eyed penguin since the study (Hunter *et al.*, 2016). The effect of chronic *Plasmodium* spp. infections in wild penguins remains unclear globally, with many authors suggesting *Plasmodium* spp. to be a significant threat to the conservation of penguins due to the known susceptibility of penguins to infection (Brossy *et al.*, 1999, Jones and Shellam, 1999, Tompkins and Gleeson, 2006, Levin *et al.*, 2009, Meile *et al.*, 2013, Grilo *et al.*, 2016). Modelling by Meile *et al.* (2013) to assess the impact of *Plasmodium* spp. infection in wild Galapagos penguins showed decreased persistence of the species due to the likely mortality from infection from *Plasmodium* spp. infection.

Avian malaria is an emerging disease in New Zealand with a growing prevalence and host range, that will continue to increase with the effects of climate change (Schoener *et al.*, 2014). This study offers the first evidence of *Plasmodium* spp. prevalence in wild yellow-eyed penguins surveyed by PCR, and showed an

apparent absence of infection in wild birds on Enderby Island. If the sub-Antarctic population of yellow-eyed penguins is truly free from avian malaria the effects of climate change this parasite may be one of the limitations to the yellow eyed penguins expanding into its more northerly New Zealand South Island habitat. Avian malaria has previously caused the extinction of Hawaiian avifauna and continues to drive the distribution of forest bird species on this island (Fortini *et al.,* 2015). With warming temperatures in the sub-Antarctic islands (Richard *et al.*, 2013), avian malaria could become established in this previously naïve ecosystem, which has had devastating consequences in wild avian populations historically (Van Riper III *et al.*, 1986, Fortini *et al.*, 2015). The study also demonstrated a significant difference in the prevalence of avian malaria in wild yellow-eyed penguins compared to those in rehabilitation, suggesting a need to implement vector prevention and treatment strategies in rehabilitation settings. Further investigation into the host-pathogen dynamics of avian malaria in yellow-eyed penguins is critical to inform the future conservation efforts of this unique and endangered species.

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CHAPTER THREE

Eimeria in wild yellow-eyed penguins: Investigation into prevalence and pathogen load, and morphologic characterisation of a novel *Eimeria* species

CHAPTER THREE – *EIMERIA* IN WILD YELLOW-EYED PENGUINS: INVESTIGATION INTO PREVALENCE AND PATHOGEN LOAD, AND MORPHOLOGIC CHARACTERISATION OF A NOVEL *EIMERIA* SPECIES

3.1 Introduction

Coccidia are obligate intracellular protozoans in the phylum Apicomplexa that infect all classes of vertebrates. *Eimeria* spp. are identified based on the morphological appearance of the sporulated oocyst and have a direct faecal-oral life cycle (Yabsley, 2008). The oocysts are resistant in the environment, with the majority of *Eimeria* spp. infecting intestinal or renal tubular epithelial cells with few exceptions (Yabsley, 2008). Coccidia infections in wild birds rarely cause disease under normal habitat conditions. However, outbreaks of disease and mortality have been documented in wild birds when conditions cause exposure to high numbers of oocysts in hosts with poor immunity, due to stress, crowding or concomitant disease (Harrigan, 1992, Yabsley, 2008, Morgan *et al.*, 2014, Alley and Gartrell, 2019). Although overt disease and mortality events occur rarely in wild bird populations, some authors have reported subclinical effects of coccidiosis on wild birds, such as reductions in feather condition, reproduction and increased susceptibility to predation (Pap *et al.*, 2013, Watson, 2013, Knight *et al.*, 2018). In addition, coccidiosis in some wild birds can exert a population level impact, by significantly reducing the survival of offspring (Bertram *et al.*, 2015).

There is little known about the impacts of parasites like coccidia on free-living seabird populations, and there is growing evidence to suggest that changing environmental conditions can significantly alter the impact of parasites on wild seabirds (Khan *et al.*, 2019). Therefore, further research is required to understand host-parasite interactions in wild seabird populations, especially in the face of ongoing climatic and anthropogenic changes to global seabird habitats (Bestley *et al.*, 2020).

Global penguin populations are facing the threats of habitat degradation, pollution and interactions with fisheries, with the effects of climate change likely to impose further pressure on the terrestrial and marine ecosystems (Trathan *et al.*, 2015). Knowledge of the diseases affecting wild penguins is scarce, but the declining and isolated populations of some endangered species of penguin are thought to be particularly at risk of changes to host-pathogen dynamics (Trathan *et al.*, 2015). The significance of coccidian parasites in free-living penguin populations remains unclear. A number of studies have reported low or no burdens during faecal surveys of wild penguins, while other authors have suggested coccidiosis to be a disease of potential

significance in isolated populations (Golemansky, 2008, 2011, 2016). Studies in wild gentoo penguins (*Pygoscelis papua)*, chinstrap penguins (*Pygoscelis anarcticus)*, emperor penguins (*Aptenodytes forsteri)* and rockhopper penguins (*Eudyptes chrysocome)* found no evidence of coccidia in all faecal samples (Karesh *et al.*, 1999, Vidal *et al.*, 2012, González-Acuña *et al.*, 2013, Kleinertz *et al.*, 2014). Coprological surveys of wild Galapagos penguins (*Spheniscus mendiculus*) found a 14.4% prevalence of unsporulated oocysts that could be identified to genus (Carrera-Játiva *et al.*, 2014). However, Golemansky (2011) suggests that coccidia could be a pathogen of significance to penguins after surveys of three wild *Pygoscelis* spp. populations over 6 years showed a prevalence of 35% (n = 360), with a high pathogen load (80-220 oocysts per microscopic field at 150x magnification) in 20 samples.

Furthermore, renal and intestinal coccidiosis has been reported in little penguins (*Eudyptula minor)* in Australia and New Zealand of varied severity. Infections have been reported more commonly in juvenile little penguins, with mostly mild and localised lesions (Obendorf and McColl, 1980, Alley, 2002, Cannell *et al.*, 2016). However, severe pathology due to renal and intestinal coccidiosis has been described in wild little penguins, secondary to mortality events associated with environmental stressors and changes in food supply (Obendorf and McColl, 1980, Harrigan, 1992, Van Rensburg, 2010). Despite these reports of severe pathology, and faecal samples from five penguin species positive for *Eimeria* spp. or *Isospora* spp., morphological descriptions of coccidial oocysts are limited or unavailable (Ranum and Wharton, 1996, Van Rensburg, 2010, Carrera-Játiva *et al.*, 2014, Golemansky, 2016). There has been only one coccidia species infecting penguins formally described, the species *Eimeria pygosceli* infecting wild gentoo penguins (*Pygoscelis papua)*, Adelie penguins (*Pygoscelis adeliae)* and chinstrap penguins (*Pygoscelis anarcticus)* (Golemansky, 2003). The significance of coccidian parasites in wild penguin populations remains unclear, but there is evidence that coccidiosis can cause severe pathology, especially when infection is exacerbated by environmental stressors.

Information regarding the prevalence or significance of coccidia infections in yellow-eyed penguins (*Megadyptes antipodes*) is scarce. There have been two reports of *Eimeria* sp. oocysts detected in the faeces of wild yellow-eyed penguins from mainland New Zealand. Ranum and Wharton (1996) reported 35% of samples were positive for *Eimeria* oocysts (n = 20) in wild yellow-eyed penguin faecal samples, with parasite burdens ranging from 2-265 oocysts/gram. In addition, one bird was also infected with 8402 oocysts / gram of oocysts that could not be sporulated. No morphological description of the *Eimeria* species seen in this study was provided. McDonald (2003) reported 18.5% prevalence (n=65), in wild yellow-eyed penguin faecal samples collected, with mean oocyst counts of 223.2 +/- 59.6 oocysts / gram. No detailed morphological description of the *Eimeria* species was reported, apart from a description of the mean oocyst length of 40.22μm +/- 0.78μm and width of 31.25μm (+/- 0.83μm). No pathology due to intestinal coccidiosis has been

described, but renal coccidiosis has been noted sporadically as an incidental finding histologically in juvenile yellow-eyed penguins (Alley *et al.*, 2004). Very little is known about coccidial parasites in the yellow-eyed penguin, and further research is required to understand the significance of these parasites to this threatened species of penguin.

The yellow-eyed penguin is an endangered species endemic to New Zealand, that has seen significant ongoing decline of the population over the last 30 years. The population is comprised of two genetically distinct and geographically isolated sub-populations, the mainland population and the sub-Antarctic population (BirdLIfe International, 2018). The mainland population includes approximately 580-780 breeding pairs along the South East coastline of the South Island of New Zealand, and Stewart Island (McKinlay, 2001, Massaro and Blair, 2003, Seddon *et al.*, 2013). The sub-Antarctic population is estimated to comprise 68-79% of the total population, with approximately 444-922 pairs in the Auckland Islands (Muller *et al.,* 2020), and 350-540 breeding pairs on Campbell Island (Moore, 1992, Moore, 2001). The mainland population has been studied extensively and is facing a number of terrestrial stressors including habitat degradation, heat stress, predation and human disturbance that the sub-Antarctic population is not exposed to (Seddon and Darby, 1990, McKay *et al.*, 1999, Moore, 2001, Seddon *et al.*, 2013, Argilla, 2015, Clark *et al.*, 2015). Due to the isolation of the sub-Antarctic birds the population the dynamics of this major sub-population remain more unclear. A recent survey between 2012-2017 suggests that the overall population trend of yellow-eyed penguins on the Auckland Islands is stable though, despite large annual fluctuations in the population (Muller *et al.,* 2020). Disease outbreaks have been observed sporadically in both sub-populations, and recent work has suggested potential differences in pathogen prevalence between the two populations (Argilla *et al.*, 2013). In addition, different patterns of mortality have been observed between the mainland and sub-Antarctic populations, with significant differences in the environmental risk factors associated with the mortality (Argilla, 2015). The yellow-eyed penguin is one of the most endangered species of penguin in the world with two distinct populations occupying vastly different habitat ranges and exposed to different ecological stressors. The host-pathogen dynamics of parasites infecting the yellow-eyed penguin remain unclear, and further research is required to understand these and hence inform future conservation efforts.

The aim of this study was to examine the potential differences in host-pathogen dynamics between the two wild populations of the endangered yellow-eyed penguin; the mainland population, and the sub-Antarctic population. This was performed by examining faecal samples to determine the prevalence and pathogen load of *Eimeria* spp. within the wild populations. Coccidia are known to be of low clinical significance in wild populations in the absence of stress or intercurrent disease, but were expected to be at detectable prevalences within the populations as a host-adapted parasite. Therefore, *Eimeria* species were selected as

the study pathogen to potentially detect the impact of stressors on the two distinct wild populations of yellow-eyed penguins. In addition, no previous description of the *Eimeria* oocysts detected in yellow-eyed penguin faeces has been reported, and so this work aims to provide the first formal morphometric description of sporulated oocysts.

3.2 Materials and method

3.2.1 Study sites

Yellow-eyed penguins were sampled from wild sites to represent the mainland and sub-Antarctic populations (**Figure 3.1**). Adult birds were sampled from the wild of south eastern Otago (45.8667° S, 170.6667° E) and the Catlins Coast (46.4636° S, 169.1972° E) to represent the mainland population of the South Island of New Zealand. Birds were also sampled from Rocky Ramp at Enderby Island, part of the Auckland Island archipelago, to represent the sub-Antarctic population of birds (50°29'45"S 166°17'44"E).

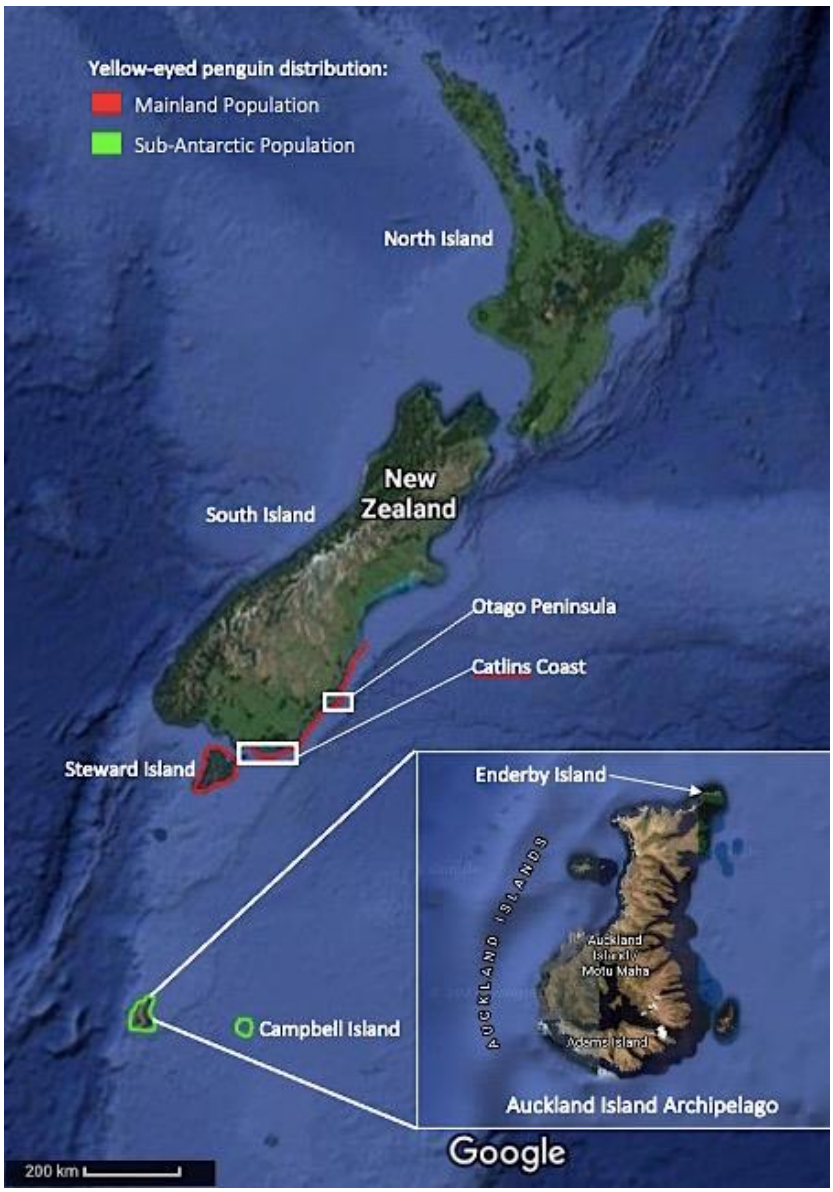

Figure 3. 1 Population distribution of yellow-eyed penguins (*Megadyptes antipodes)* and study sites

3.2.2 Collection of faecal samples

Adult wild yellow-eyed penguins were opportunistically selected on Enderby island (n = 47) during the breeding season between November 2015 – January 2016, and from the Otago Peninsula and Catlins coast (n = 41) between February 2017 – June 2017. Faecal samples were collected from each bird voided during handling, or via gentle digital expression of the cloaca.

All wild birds captured were given a physical examination and weighed at capture. Morphometric measurements were taken to allow sexing of birds via the combined crown and beak length, and the foot length, and were confirmed via the database of information for mainland birds where possible. A body condition score based on pectoral muscle mass was subjectively recorded for each bird using a scale of 1-9 adapted from the scale by Clements and Sanchez (2015). Each bird was also scanned for a microchip, and had a passive integrated transponder placed subcutaneously if no microchip was already present. Faecal samples were stored in individually labelled sterile containers and stored in a cool dry location until they could be refrigerated at 4°C until processing. Due to the remote sampling location of both populations the faecal samples were stored at the environmental conditions for weeks prior to having access to refrigeration for storage.

3.2.3 Faecal floatation technique

A standard veterinary protocol for faecal floatation was used to analyse the yellow-eyed penguin faecal samples (Zajac and Conboy, 2012). Between 0.2 - 1g of faeces was suspended in 33% zinc sulphate solution (S.G. 1.2) and agitated until all pieces of plant material and environmental substrate were separated visually. The solution was then strained through a fine nylon mesh sieve into a 15 mL centrifuge tube. The test tube was filled with additional zinc sulphate until a shallow fluid meniscus formed at the top of the tube. A coverslip was placed on top of the test tube ensuring complete contact between the cover slip and the fluid meniscus of the suspension. The samples were centrifuged in a swinging centrifuge at 177 g for 5 minutes. The coverslip was then removed from the tube to be placed on a microscope slide and examined under light microscopy (Olympus CX41) for the presence of coccidia. The entire coverslip was examined for each sample, and oocysts were counted at 300x magnification to report the pathogen load as oocysts / gram of faeces. For samples with very high oocyst counts (> 20 oocysts per 20x field), a central transect of the coverslip was used to determine the pathogen load instead of counting the whole coverslip. This central transect was one field of view wide, and the resulting number of oocysts counted was multiplied by 25 to give the total count, based on previous calibration of the microscope.

3.2.4 Oocyst morphological examination and measurement

All faecal samples with identifiable oocysts present had sporulated by the time of examination, apart from seven positive samples collected from Enderby Island birds. A one-gram sub-sample of each of these unsporulated faecal samples was mixed in a suspension of faeces and sterile water, or faeces and potassium dichromate (2% w/v) and stored at 25°C for seven days. The samples were assessed for sporulation by examining a drop of the sub-sample under light microscopy from day 3. All seven samples failed to sporulate under these conditions.

To prepare samples for morphological examination of sporulated oocysts, the oocysts were initially concentrated by the above described floatation technique. The coverslip was placed onto the microscope slide, and then allowed to sit at room temperature for 10-15 minutes until the zinc sulphate solution had crystallised around the periphery of the coverslip. The edges of the coverslip were then sealed with clear, quick set nail varnish (Sally Hansen Rapid Set) to protect the sample from desiccation and increase the time available to examine and measures the sporulated oocysts in the sample.

The samples with sporulated oocysts of sufficient quality were examined under light microscopy with a Leica DM750 at 1000x magnification under oil immersion, to document the morphological characteristics of the oocysts. The sporulated oocysts of some samples were degraded or ruptured, and so intact oocysts were selected for measurements. Fully sporulated oocysts were positioned in a longitudinal transverse plane to be captured by both still and video images with Leica ICC50 W (Leica Microsystems, Germany). Measurements (μm) and observations were documented for each suitable oocyst using the method described by Duszynski and Wilber (1997). For each oocyst measured numerical averages were calculated, as not all sporocysts or sporozoites were measurable in each oocyst. In addition, morphometric ratios of oocyst length:width and sporocyst length:width were calculated. A composite drawing was created with Adobe Illustrator of a single representative oocyst using a video progression through focus planes as a reference.

3.2.5 Statistical analysis

The apparent prevalence of *Eimeria* and 95% confidence interval was calculated using the Epitools software following the Wilson binomial approximation (Brown *et al.*, 2001). The difference in prevalence between the two locations was compared by Chi-square analysis (Preacher, 2001). A Mann-Whitney U analysis was used to assess for statistically significant differences in the pathogen loads between the two sample populations. A non-parametric analysis (Mann-Whitney U) was also performed to analyse differences in body condition scores between infected and non-infected birds between the two locations. In addition, ANOVA significance testing was used to assess for variation in the morphological measurements of oocysts from each sample location. A linear regression analysis was used to assess for agreement in the morphometric

measurements of oocysts, and the possible presence of more than one species of *Eimeria* in the faecal samples collected. Linear regression analysis was also used to explore the relationship between coccidia pathogen load and the physical parameter of body condition score.

3.3 Results

3.3.1 Infection prevalence based on faecal floatation in wild birds

Faecal samples of 47 yellow-eyed penguins from Enderby Island and 41 wild penguins from the South Island of New Zealand were collected and tested by faecal floatation for the presence of coccidial oocysts. The apparent prevalence of *Eimeria* sp. in wild yellow-eyed penguins on Enderby Island was 76.6% (95% CI 62.78-86.4%), with 36/47 samples positive for *Eimeria* on examination (**Figure 3.2**). The apparent prevalence of *Eimeria* sp. in wild yellow-eyed penguins on the South island of New Zealand was 58.54% (95% CI 43.37-72.24%) with 24/41 samples positive for *Eimeria.* There was weak evidence (χ^2 = 3.292, p = 0.07) for a difference in the prevalence of infection between the two populations.

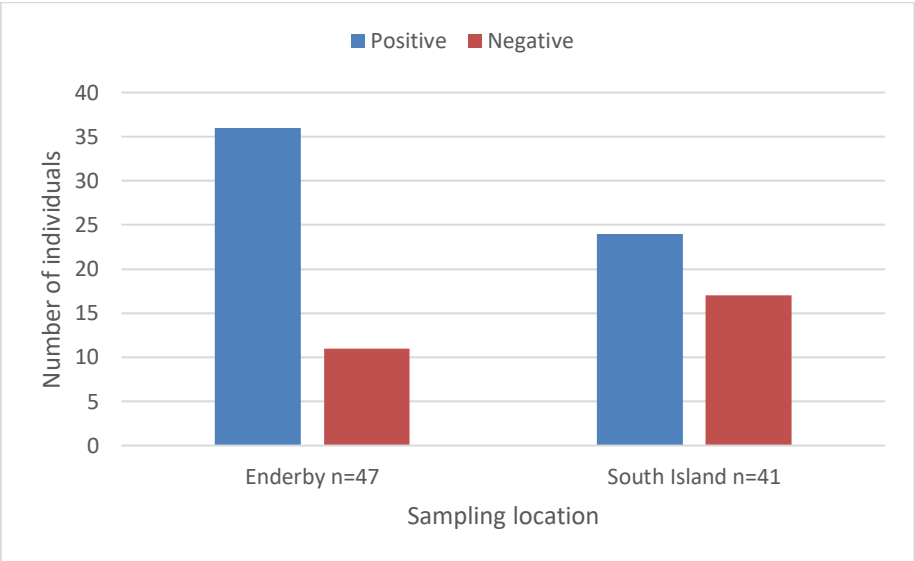

Figure 3. 2 Coccidian presence in faecal samples collected from wild yellow-eyed penguins from Enderby Island and the South Island of New Zealand. Individuals with oocysts in blue, and without oocysts in red at each sample location.

3.3.2 Geographic variation in pathogen load

The mean pathogen load (measured as oocysts per gram) of oocyst shedding in all the samples from wild yellow-eyed penguins on Enderby island was 9723 oocysts/gram (s.e. = 5831 occysts/gram), with a maximum pathogen load in one bird of 218,750 oocysts/gram (**Figure 3.3**). The mean pathogen load in all the samples from wild yellow-eyed penguins from the South Island of New Zealand was 1050 oocysts/gram (s.e. =

398 oocysts/gram), with a maximum pathogen load in one bird of 14,750 oocysts/gram (Table 3.1). An analysis of coccidial oocysts counts from each sample location showed there was no evidence for a difference in pathogen load between the wild birds of Enderby Island and those from the South Island of New Zealand (p = 0.1991).

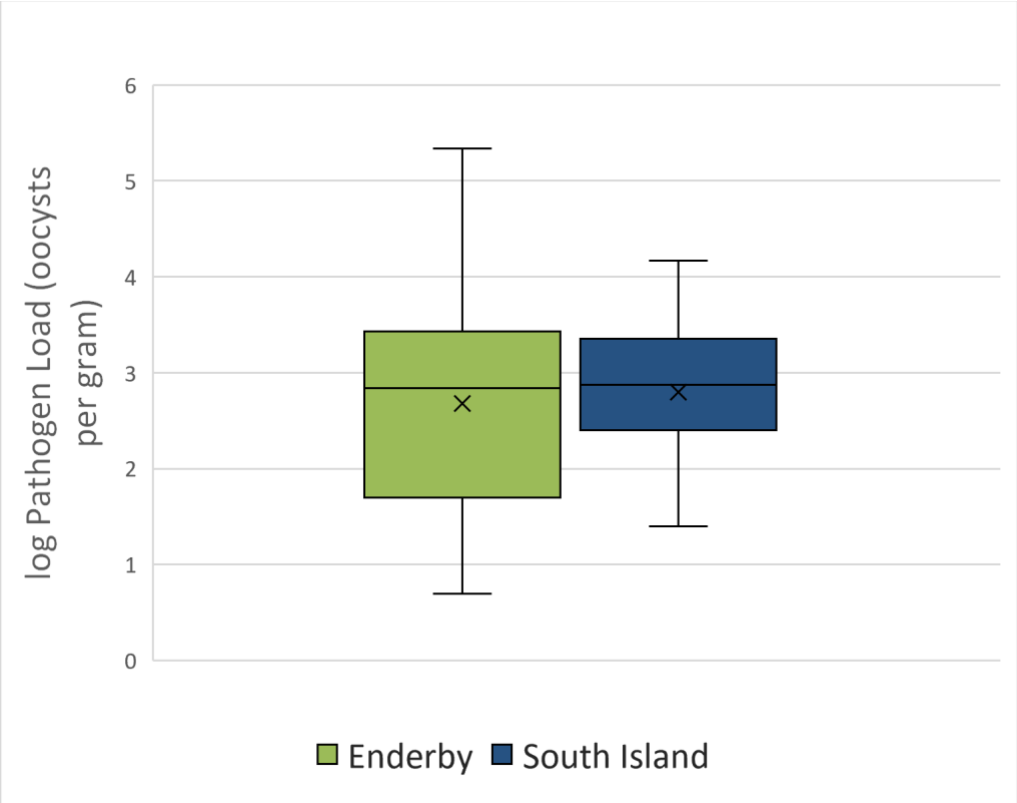

Figure 3. 3 Log transformed coccidian oocyst counts in faecal samples collected from wild yellow-eyed penguins in 2017 from Enderby island and the South Island of New Zealand. Pathogen load measured in oocysts per gram of faeces, where the x represents the mean pathogen load in each location, the centre line represents the median pathogen load, the box represents the first and third quartile of the pathogen load and whiskers show the minimum and maximum loads at each location.

3.3.3 Body condition score variation with *Eimeria* spp. infection status and pathogen load

The mean body condition score (BCS) for yellow-eyed penguins positive for *Eimeria* spp. infection across both sample locations was 3.9 (s.e = 0.15), and for negative birds across both locations was also 3.9 (s.e = 0.22) (Figure 3.4). There was no evidence for a difference in body condition score or weight between *Eimeria* spp. positive and negative birds when the results of both locations were combined (p=0.8973).

The mean body condition score for negative birds sampled from Enderby Island was 3.6 (s.e = 0.21), while the mean BCS for negative birds sampled on the South Island of New Zealand was 4.2 (s.e = 0.34), with no evidence for a statistical difference between the two groups (p = 0.332) (Table 3.1). However, there was strong evidence for a statistical difference between the mean body condition scores of *Eimeria* sp. positive birds on Enderby Island compared to positive birds sampled on the South Island of New Zealand (p= <0.0001). The mean body condition score for positive birds on Enderby Island was 3.4 (s.e = 0.09), while the mean BCS for positive birds on the South Island was 4.7 (s.e = 0.27) (Figure 3.4).

No evidence of an association was found between pathogen load and body weight (R^2 = 0.012, F= 0.938, p = 0.336) in either study population.

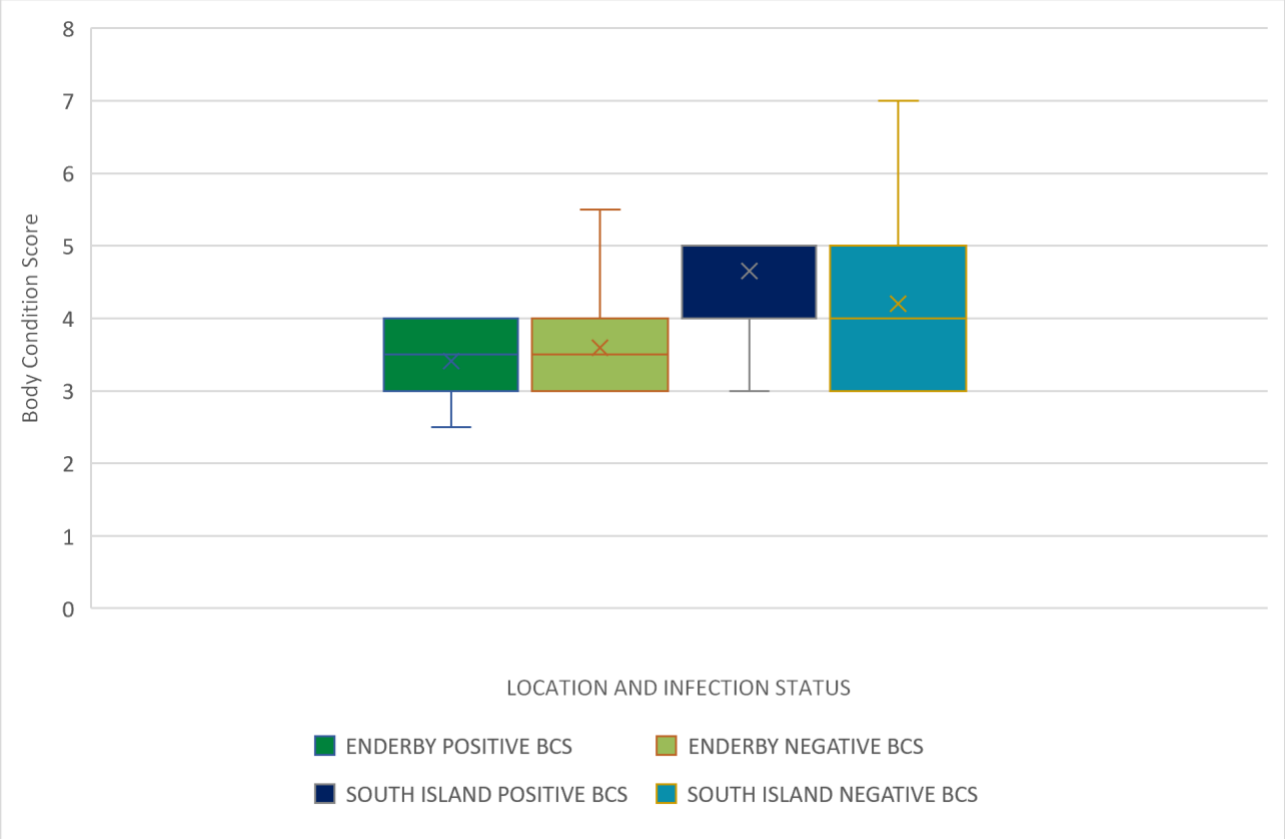

Figure 3. 4 Body condition scores of wild yellow-eyed penguins identified as either positive or negative for coccidian oocysts in faecal samples from Enderby island and the South Island of New Zealand. The body condition score mean represented as an x, median as the centre line, and maximum and minimum scores by the limits of each plot.

Table 3. 1 *Eimeria* spp. prevalence and pathogen loads in faecal samples collected from wild yellow-eyed penguins sampled from Enderby Island and the South Island of New Zealand, and mean body condition scores (BCS) for positive and negative birds at each sample location.

	Enderby Island (n=47)	**South Island (n=41)**	**All (n=88)**	**Sig**
Prevalence (%)	76.6	58.54	68.18	P = 0.07

Prevalence Lower 95% CL (%)	62.78	43.37	57.87	
Prevalence Upper 95% CL (%)	86.4	72.24	76.98	
Mean FEC ± standard deviation (oocysts per gram)	9723 ± 39978	1050 ±2548	5682 ±29444	P = 0.1991
Maximum pathogen load (oocysts per gram)	218,750	14,750	218,750	
Mean BCS Coccidia Positive Birds ± standard deviation	3.4 ± 0.5	4.7 ± 1.3	3.9 ± 1.1	**P = <0.0001**
Mean BCS Coccidia Negative Birds ± standard deviation	3.6 ± 0.7	4.2 ± 1.3	3.9 ± 1.1	P = 0.332

Pathogen loads and body condition scores reported as Mean ± standard deviation, with ANOVA significance (p-value <0.05) interactions between the different populations of yellow-eyed penguins sampled.

3.3.4 Evaluation for consistency among oocysts

A linear regression of oocyst width (μm) over oocyst length (μm) was performed to evaluate whether the oocysts measured represent a single species across the two sampling locations, and distinct populations of yellow-eyed penguins. The calculated regression is described in Figure 3.5 below.

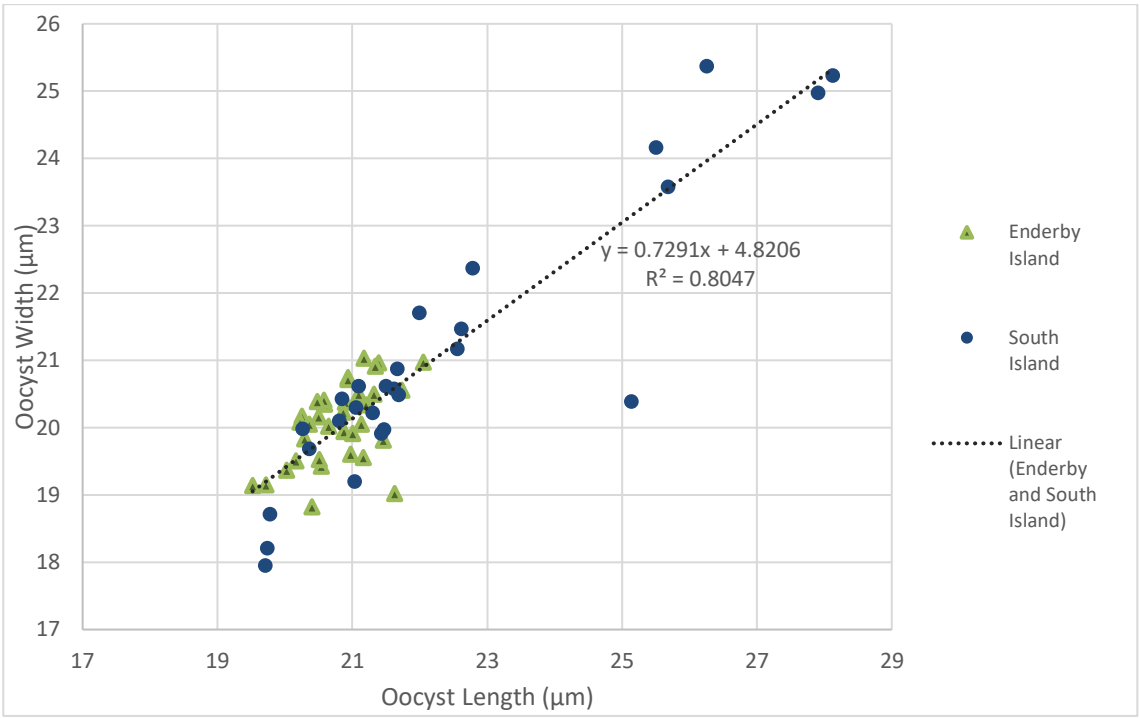

Figure 3. 5 Linear regression illustrating the variation in oocyst width and length. Data points from different sampling locations indicated, Enderby Island (triangles) and South Island of New Zealand (dots)

The R^2 value calculated for the linear regression for all oocysts measured at both sampling sites was 0.80, and therefore above the standard $R^2 > 0.5$ used to indicate variation within a single population of oocysts (Berto *et al.*, 2014). This result suggests only a single species of *Eimeria* was present at both sampling locations, with only slight variations in the oocyst morphometrics.

3.3.5 Morphological oocyst measurements

Table 3.2 details the measurements taken to complete the morphological description of coccidian species recovered from the yellow eyed penguins. These measurements include the oocyst wall thickness measured at the equator, and the length and width of the oocyst, sporocysts and sporozoites. The length width ratios for the oocyst and sporocyst were then calculated based on the measurements. The data below is presented as mean ± standard deviation for both the South Island of New Zealand and Enderby island sampling sites, and the total oocysts measured. ANOVA significance testing was used to examine for variation in the oocyst morphometrics between the two sampling sites. There was statistically significant variation between the oocysts collected from Enderby Island and the South Island of New Zealand in all measurements apart from the oocyst wall thickness.

Table 3. 2 Oocyst measurements taken from coccidial oocysts extracted from faecal samples collected from wild yellow-eyed penguins sampled from Enderby Island and the South Island of New Zealand

	South Island	Enderby Island	Total	Sig.	Eta²

	(n = 27)	(n = 35)	(n = 62)		
Oocyst Length (μm)	22.37 ±2.42	20.80 ±0.56	21.49 ±1.81	**0.0004**	**0.188**
Oocyst width (μm)	21.04 ±2.02	20.06 ±0.59	20.49 ±1.47	0.0083	0.111
Oocyst L:W Ratio	1.06 ±0.05	1.04 ±0.03	1.05 ±0.04	0.0083	0.105
Wall thickness (μm)	0.34 ±0.06	0.36 ±0.06	0.35 ±0.06	NS	0.008
Sporocyst length (μm)	12.17 ±1.23	10.54 ±0.50	11.20 ±1.18	**< 0.0001**	**0.461**
Sporocyst width (μm)	7.16 ±1.00	6.80 ±0.35	6.95 ±0.72	**0.0009**	0.062
Sporocyst L:W Ratio	1.71 ±0.23	1.56 ±0.08	1.63 ±0.18	**0.0007**	**0.175**
Sporozoite length (μm)	10.67 ±1.48	6.65 ±0.43	8.48 ±2.26	**< 0.0001**	**0.786**
Sporozoite width (μm)	3.80 ±0.51	4.29 ±0.33	4.04 ±0.50	**< 0.0001**	**0.245**

Mean ± standard deviation, with ANOVA significance and Eta^2 (effect size) for significant interactions between location of sampling and the given measurement (P-value <0.05, or Eta^2 >0.14). NS = not significant.

3.3.6 Morphological Description

Type host: *Megadyptes antipodes* (Aves, Sphenisciformes, Spheniscidae)

Parasite: *Eimeria* novel morphotype P (**Figure 3.6, 3.7**)

Type locality: Rocky Ramp at Enderby Island, part of the Auckland Island archipelago (50°29'45"S 166°17'44"E).

Other localities: South eastern Otago (45.8667° S, 170.6667° E) and Catlins Coast (46.4636° S, 169.1972° E) South Island of New Zealand.

Prevalence: Apparent prevalence on Enderby Island is 76.6% (95% CI 62.78-86.4), while the apparent prevalence on the South Island of New Zealand is 58.54% (CI 43.37-72.24)

Sporulation: Unknown. Stored at environmental temperatures at sampling location for 1-8 weeks, and then refrigerated at 4°C until processing

Site of infection: Unknown.

Description of sporulated oocysts, with all measurements presented as mean ± standard deviation (range):
Tetrasporic dizoic sporulated oocysts characteristic of the genus *Eimeria*. The shape of the oocyst was subspherical, L:W ratio was 1.05 ± 0.04 (1.01 – 1.09). Oocyst dimensions were 21.49 ± 1.81 x 20.49 ± 1.47 (19.68 - 23.3 x 19.02 – 21.96) µm. The wall was smooth, single layered, fragile and of thickness 0.35 ± 0.06 (0.29 - 0.41) µm. The micropyle was absent. The oocyst residuum was present. No polar granule was present.

Description of sporocysts and sporozoites:

Sporocysts were elongate 11.20 ± 1.18 x 6.95 ± 0.72 (10.02-12.38 x 6.23 – 7.67) µm and L:W ratio 1.63 ± 0.18 (1.45 – 1.81). A granular sporocyst residuum was present, but a Stieda body, sporopedia, sub-Stieda body, and sporocyst sutures were all absent. Each sporocyst contained two crescent-shaped sporozoites 8.48 ± 2.26 x 4.04 ± 0.50 (6.22 – 10.74 x 3.54 – 4.54) µm. One large spherical posterior refractile granule was present within each sporozoite, and a smaller anterior refractile granule.

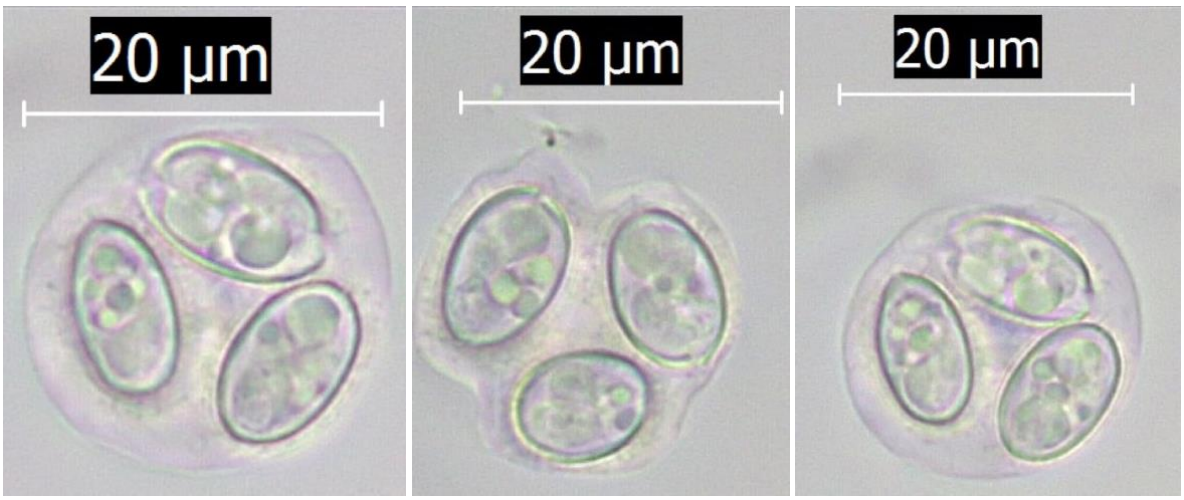

Figure 3. 6 Micrographs of sporulated oocysts (x100) of a novel *Eimeria* sp. isolated from yellow-eyed penguin hosts

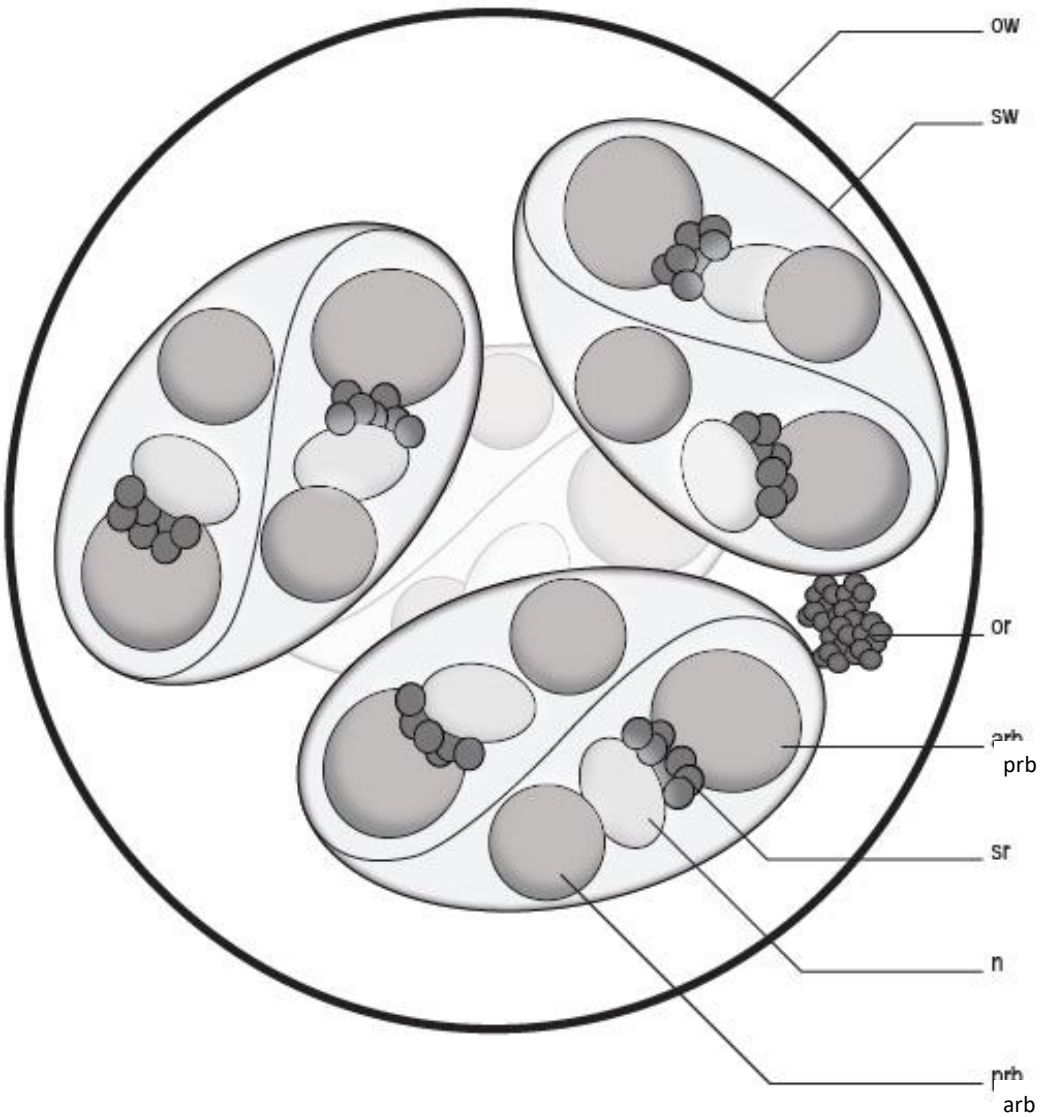

Figure 3. 7 Annotated schematic drawing of a sporulated oocyst of a novel *Eimeria* sp. morphotype isolated from a yellow-eyed penguin. Labels indicating oocyst wall (ow), oocyst residuum (or), sporocyst wall (sw), sporocyst residuum (sr), sporozoite nucleus (n), posterior refractile body (prb), anterior refractile body (arb).

3.4 Discussion

This study provides the first formal morphological description of an *Eimeria* protozoa from a yellow-eyed penguin host. Golemansky (2003) provides the only other description of an *Eimeria* species oocyst isolated from a member of the Sphenisciformes*, Eimeria pygosceli*. The novel *Eimeria* morphotype reported here can be differentiated from *Eimeria pygosceli* in a number of ways. Firstly, *Eimeria pygosceli* has a thick double wall (1.5-1.8 µm) compared to the thin single layered wall (0.29-0.41 µm) of the yellow-eyed penguin *Eimeria*. In addition, the length and width of *Eimeria pygosceli* oocysts (28-32 x 24-28 µm) are larger than the dimensions of the novel *Eimeria* (19.68 - 23.3 x 19.02 – 21.96 µm). The dimensions of the sporocysts of *Eimeria pygosceli* (11-13 x 6-7 µm) reported are similar to those of the novel *Eimeria* of the yellow-eyed penguin (10.02-12.38 x 6.23 – 7.67 µm). However, unfortunately only an image of an unsporulated *Eimeria pygosceli* oocyst could be found for comparison. Molecular analysis was beyond the scope of this study but is required to complete the description of the novel *Eimeria* species reported here and confirm that the oocysts are distinct from *Eimeria pygosceli.*

Due to the isolation of the sampling sites and absence of refrigeration, the faecal samples were stored at the environmental temperature of the habitat for weeks before laboratory processing. Under these conditions most of the samples (81/88) sporulated successfully, but it is possible the inability to refrigerate the samples shortly after collection may have altered the dimensions of the oocysts. Storage conditions have been previously noted to affect oocyst morphology, and so this must to be considered in review of the results reported here (Duszynski and Wilber, 1997, Berto *et al.*, 2014). If we accept the criteria of regressing oocyst width against length (Berto *et al.*, 2014), then it is possible only a single novel species of *Eimeria* was recovered from birds in both locations. It is worth noting that there was strong evidence for morphological differences in the measurements of the oocysts recovered between the two sample populations. One possible explanation for this variation is the difference in environmental conditions between the two sample sites. As stated earlier, due to the isolation of the sampling locations the faecal samples were held at environmental temperatures before being refrigerated, and so the differences in the climate of the two habitats may have altered the oocysts accounting for this variation. Another possible explanation is that there was a different species of *Eimeria* isolated from each location and there is geographic isolation and genetic distinction between the two yellow-eyed penguin populations sampled in this study. However, I contend that the regression analysis combined with the strong consistency in morphology among the oocysts examined suggests only a single species of novel *Eimeria* was present in both populations of yellow-eyed penguins sampled. Molecular analysis will be needed to confirm this hypothesis.

This study aimed to examine potential differences in host-pathogen dynamics between the two distinct populations of the endangered yellow-eyed penguin, the sub-Antarctic and the mainland populations. These two populations are genetically and geographically distinct, occupy unique and distinctive habitat ranges, and face stressors specific to the environment of the population centre (Boessenkool *et al.*, 2009b, 2010, Seddon *et al.*, 2013, Argilla, 2015). Despite the ecological differences between the isolated populations, there was only weak evidence for a difference in the apparent prevalence of *Eimeria* in the faecal samples of wild yellow-eyed penguins, or in the pathogen load between the two study groups. These results show that the *Eimeria* species identified in this study is capable of successful replication and transmission between penguin hosts in an array of environmental conditions. *Eimeria* species are generally considered to be host specific, and so may have co-evolved with the yellow-eyed penguin to allow survival in the different climatic conditions of the population centres. The yellow-eyed penguin only recently colonised the South Island after the extinction of another penguin species *Megadyptes whaitaha* (Boessenkool *et al.*, 2009a). If only a single species of *Eimeria* was identified in both populations, it is possible that the pathogen co-evolved with the yellow eyed penguins of the sub-Antarctic and was transported to the mainland of New Zealand with it's hosts relatively recent colonisation (Boessenkool *et al.*, 2009a).

The prevalence and pathogen load for both locations in this study were higher than previous reports of coccidial oocysts collected in the faeces of yellow-eyed penguins, and wild Sphenisciformes globally. The prevalence and pathogen load both in the Enderby Island and South Island populations sampled were higher than in previous reports (Ranum and Wharton, 1996, McDonald, 2003). In addition, the prevalence and pathogen loads identified in this study are also higher than results of faecal coccidia surveys in wild penguin species in other areas. Surveys of wild Galapagos penguins found 14.4% prevalence of unsporulated oocysts in faecal samples of unknown identification (Carrera-Játiva *et al.*, 2014). Golemansky (2011) surveys of wild *Pygoscelis* spp. populations showed a prevalence of 35% (n = 360), with a high pathogen load (80-220 oocysts in one microscopic field at magnification of 150x) in 20 samples. Golemansky (2011) suggested that the high pathogen load observed in these 20 birds was an indirect indication of the pathogenic role of coccidians in the free-living penguin populations examined. The higher prevalence and pathogen load observed in the yellow-eyed penguin samples of my study could indicate the pathogenic potential of *Eimeria* for the yellow-eyed penguin as Golemansky (2011) suggested. However, another possibility is that the yellow-eyed penguin has co-evolved with this *Eimeria* species and developed tolerance strategies to facilitate pathogen transmission and survival, with limited effect on the hosts (Henschen and Adelman, 2019).

The results of this study showed strong evidence for a difference in the mean body condition of yellow-eyed penguins infected with the novel *Eimeria* morphotype between the two study populations. The mean

body condition score of infected birds was significantly lower on Enderby Island when compared to positive birds from the mainland population of the South Island of New Zealand. These results suggest that the host-pathogen dynamics of the novel *Eimeria* morphotype may vary between the two study populations, with the potential for an increased clinical cost of coccidial infection in the sub-Antarctic birds. However, no real estimation of the clinical impact of infection should be made without further research as the body condition of birds can be influenced by an array of confounding factors. Of particular note is the difference in seasonal sampling time between the two study sites. Due to the climatic conditions of Enderby Island, penguins were sampled during November and January, while the South Island birds were sampled between February and June. Due to this difference in sampling timing, the Enderby birds were sampled during the guard and post-guard stages of chick rearing, while the South Island birds were sampled during the cycles of chick fledging, and pre and post moult (Seddon *et al.*, 2013). Therefore, it is likely that the differences in body condition is at least in part due to differences in cyclic physiological demands on the hosts at the times of sampling. Furthermore, there was no evidence of coccida presence or pathogen load affecting bodywight or body condition score when the samples were examined as a whole, suggesting infection may have little clinical effect on this parameter.

The endogenous site of development (tissue tropism) for this novel *Eimeria* morphotype and its pathogenicity remain unclear. There is only one histological report of coccidia infection in yellow-eyed penguins consisting of renal coccidiosis identified as an incidental finding in juvenile birds (Alley *et al.*, 2004). There have been no reports of intestinal pathology due to coccidiosis in yellow-eyed penguins, despite ongoing intensive monitoring of the mainland population. Therefore, it is unlikely the *Eimeria* species identified in this study is a pathogen causing significant morbidity or mortality under normal conditions. However, the subclinical or population level effects of coccidial infections on wild avian populations is unclear. The significant difference in body condition scores of infected birds between the two genetically and geographically distinct study populations suggests possible differences in the host-pathogen dynamics of the novel *Eimeria* species. There is evidence that chronic infection with *Eimeria* may reduce reproductive success and survival of wild hosts (Watson, 2013). In addition, modelling has demonstrated that parasitic infections may exacerbate the negative effects of wild environmental habitat changes on free-living populations (Singer *et al.*, 2013). Furthermore, there are sporadic reports of severe lesions due to renal and intestinal coccidiosis in little penguins in Australia and New Zealand, exacerbated by unfavourable environmental conditions (Obendorf and McColl, 1980, Harrigan, 1992, Van Rensburg, 2010). Therefore, I recommend every opportunity is taken to monitor birds for faecal coccidial oocysts, and to systematically examine the intestines and kidneys of wild yellow eyed penguins using histology when suitable specimens are available. Further opportunistic monitoring is necessary to identify the tissue tropism of the novel *Eimeria* reported here, and to understand the significance of this parasite to the endangered yellow-eyed penguin population.

The high prevalence and pathogen load of *Eimeria* species in both populations of yellow-eyed penguins suggests this parasite is successful in the wild conditions. The results suggest stable and efficient transmission pathways within the population, and potentially long-established co-evolution between the *Eimeria* species identified and the yellow-eyed penguin. The apparent mild or absent clinical effect of the *Eimeria* organism on the yellow-eyed penguin provides further evidence of possible co-evolution (Henschen and Adelman, 2019). The unique ecology of the yellow-eyed penguin should be considered relation to possible transmission pathways of this novel *Eimeria* species. Yellow-eyed penguins are a non-colonial, shy species of penguin that tend to nest in sites with visual barriers to other birds chosen to maximise insulation from the heat (Clark *et al.*, 2015). In addition, they are ocean going birds that embark on long foraging trips at sea. Dilution of faecal oocysts in the ocean, makes infection with *Eimeria* during marine foraging trips unlikely. The penguins are thus likely infected during transit from the ocean back to nest sites, and around nest sites or communal areas (Ranum and Wharton, 1996). Upon return from foraging trips the penguins engage in preening and social behaviour with multiple birds accumulating in proximity at the shoreline. In some locations, penguins also take shared routes from the ocean back to nest sites. It is likely that the penguins collect oocysts deposited in the environment at nest sites or along these terrestrial routes on their feet and feathers, and are then infected orally during preening (Ranum and Wharton, 1996).

The timing of faecal sample collection must be considered in relation to the results reported here. Numerous studies have demonstrated that coccidial oocyst shedding in avian hosts varies with the time of day, showing a markedly diurnal pattern of shedding (Brawner III and Hill, 1999, Villanúa *et al.*, 2006, Berto *et al.*, 2014). Due to the endangered status of the yellow-eyed penguin, the timing of faeces collected from penguins was opportunistic to coordinate with other management or research efforts, and thereby minimise disturbance of the birds. As a result, the faecal samples could not be consistently collected at any specific time, let alone in the time of expected peak shedding. If *Eimeria* in yellow-eyed penguins follow the same diurnal shedding patterns as other avian *Eimeria* it is possible that infected birds may not shed oocysts in morning samples and may not shed consistently throughout the infection period. Therefore, if the faecal samples of this study are representative of the yellow-eyed penguin population, the true *Eimeria* prevalence and pathogen load may be higher than the results reported here. Even so, the pathogen load and apparent prevalence of *Eimeria* is still higher than previous reports of coccidial oocysts in wild Sphenisciformes globally. Collection of faecal samples at regular intervals would be required to determine if there is a temporal variation to oocyst shedding in the yellow eyed penguins.

The yellow-eyed penguin is facing a number of ongoing threats to the survival of the population, and conservation efforts to preserve this unique species involve intensive monitoring and management of the

mainland population of birds. Consequently, large numbers of birds are admitted to rehabilitation facilities annually. Further monitoring for *Eimeria* species in yellow-eyed penguins via faecal screening in rehabilitation is indicated based on the high prevalence and sometimes high pathogen load seen in the wild birds in this study. Coccidiosis is known to cause morbidity and mortality in wild birds in conditions encouraging exposure to high numbers of oocysts or depressed immunity (Yabsley, 2008, Morgan *et al.*, 2014). As the impact of this parasite on the birds remains unclear, opportunistic monitoring, especially of birds in human care, is required to understand the host-pathogen dynamics in changing conditions and to inform conservation efforts and strategies.

This study provides the first formal morphometric description of a novel *Eimeria* morphotype infecting yellow-eyed penguins. The study found no difference in the prevalence or pathogen load of the *Eimeria* between the genetically distinct and geographically isolation populations of the yellow-eyed penguin occupying mainland New Zealand and the sub-Antarctic. Only a single type of *Eimeria* was identified in both populations of wild birds. The prevalence and pathogen load of *Eimeria* was significantly higher than previous reports of coccidial oocysts in yellow-eyed penguins and free-living Sphenisciformes globally. The host-parasite relationship deserves further monitoring and investigation as the impact of this novel organism on the population remains unclear. The yellow-eyed penguin population has shown persistent decline for the last 30 years and is facing a growing number of threats with the onset of climate change. Therefore, continued investigations into host-pathogen relationships are crucial to monitor changes in disease incidence and inform ongoing conservation efforts.

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CHAPTER FOUR

General Discussion

CHAPTER 4 GENERAL DISCUSSION

Geographically distinct wildlife populations may be exposed to distinctive and unique challenges in host-pathogen dynamics. Examining the differences in these dynamics can inform predictions of a population's likely response to major climate shifts, translocations or regional colonisation. This study explored possible differences in host-pathogen dynamics between the two population centres of the yellow-eyed penguin. Models have demonstrated that parasitic relationships can exacerbate the effects of major climate shifts in wild populations (Singer *et al.*, 2013). Therefore, this study examined the prevalence and pathogen load differences of two known parasites, *Eimeria* and *Plasmodium*, within the two major geographically and genetically distinct populations of yellow-eyed penguins. In addition, the study also documented differences in the prevalence of *Plasmodium* infection between birds in rehabilitation and those in the wild.

This thesis provides the first morphological description of a novel morphotype of *Eimeria* protozoa from a yellow-eyed penguin host. *Eimeria* sp. oocysts in faecal samples of yellow-eyed penguins have been reported only twice before, with both studies failing to formally describe the oocysts seen (Ranum and Wharton, 1996, McDonald, 2003). There was strong statistical evidence for morphological differences in the novel *Eimeria* morphotype oocysts recovered from the two distinct populations of this study, however linear regression analysis suggested only a single novel type of *Eimeria* was isolated from both populations of penguins. In addition, there was only weak evidence for a difference in the apparent prevalence of *Eimeria* in faecal samples, or in the pathogen load between the two study groups of wild yellow-eyed penguins. Therefore, it is possible that the novel *Eimeria* identified in this study is a single species that co-evolved with the yellow-eyed penguins of the sub-Antarctic and was transported to the mainland of New Zealand with the recent colonisation (Boessenkool *et al.*, 2009, 2010). Molecular analysis was beyond the scope of this study but will be required to confirm this hypothesis. Alternatively, each population of yellow eyed penguins may harbour a unique species of *Eimeria.*

The prevalence and pathogen load of *Eimeria* oocysts in faecal samples reported in this study from both locations were higher than previous reports in yellow-eyed penguins, and free-living Sphenisciformes globally. These results suggest stable and efficient transmission pathways within the population, and possible long-established co-evolution between the novel *Eimeria* identified and the yellow-eyed penguin (Henschen and Adelman, 2019). However, the site of development for the novel *Eimeria* within the host and the associated costs to infected hosts remains unclear. There was a significant difference in body condition scores of infected penguins between the two distinct population centres study populations suggesting possible differences in the host-pathogen dynamics of the *Eimeria* protozoa between the two distinct population centres. There is a growing body of evidence that chronic infections with *Eimeria* may reduce survival of wild hosts (Watson, 2013), and exacerbate the negative effects of wild environmental habitat changes or stressors

(Obendorf and McColl, 1980, Harrigan, 1992, Van Rensburg, 2010, Singer *et al.*, 2013). Therefore, further monitoring is required to determine the tissue tropism of the novel *Eimeria* reported in this study, and to identify the significance of the parasite to the yellow-eyed penguin.

Penguins have a known susceptibility to *Plasmodium*, and there has been documented evidence of increasing mortality in yellow-eyed penguins due to avian malaria (Alley, 2001, Hunter *et al.*, 2016). Previous surveys for avian malaria in the yellow-eyed penguin have been contradictory though (Graczyk *et al.*, 1995, McDonald, 2003, Sturrock and Tompkins, 2007). This study offers the first report of a PCR positive result for *Plasmodium* spp. in wild yellow-eyed penguins, and documents the pathogen load of infection via LSU-RNA qPCR. The results of this study also showed good evidence for differences in the prevalence of *Plasmodium* sp. between the wild birds of the two population centres of the yellow-eyed penguin, with a higher prevalence of avian malaria in birds of the mainland population. This result may be explained by variation in the infection dynamics at the pathogen, host or vector level between the two study populations (Niebuhr *et al.*, 2016). Examples of possible habitat variations include differences in vector density, or climatic conditions between the two main population centres surveyed, as these have been shown internationally to influence the prevalence of *Plasmodium* spp. infection in wild avian populations (Szöllősi *et al.*, 2011, Fecchio *et al.*, 2013, Svensson-Coelho *et al.*, 2013, Gonzalez-Quevedo *et al.*, 2014, Fortini *et al.*, 2020). Further investigation is required to characterise the differences in host-pathogen dynamics between the two main populations of yellow-eyed penguins in relation to *Plasmodium*.

In addition, this study showed very strong evidence for a higher prevalence of *Plasmodium* spp. in yellow-eyed penguins in rehabilitation compared to wild populations. Three different strains of *Plasmodium* were identified via sequencing in the samples from birds in rehabilitation. All three strains are commonly maintained in wild New Zealand passerine populations (Sijbranda *et al.*, 2016), suggesting spill-over of infection into birds during the rehabilitation process. This is consistent with global studies on *Plasmodium* in penguin rehabilitation facilities suggest newly acquired infections are the most common source of avian malaria infection (Parsons and Underhill, 2005, Vanstreels *et al.*, 2014, Vanstreels *et al.*, 2015, Botes *et al.*, 2017). This diversity of malarial strains also suggests that host factors in response to the stress of the rehabilitation environment play a more significant role in infection and disease expression than pathogen virulence. The incidence of *Plasmodium* infection in rehabilitation penguins has the potential to cause significant morbidity and mortality as has been documented in international rehabilitation facilities (Parsons and Underhill, 2005, Vanstreels *et al.*, 2014, Botes *et al.*, 2017). The significantly higher prevalence of *Plasmodium* spp. in yellow-eyed penguins in rehabilitation compared to wild populations suggests this pathogen poses a risk to the future success of rehabilitation, and so the host-pathogen dynamics of infection in this setting deserves further research.

The research presented in this thesis offers initial insight into differences in parasite infection dynamics of the distinct population centres of the yellow-eyed penguin, but further research is necessary. The results provide a platform for future work that is necessary to truly characterise the complex host-pathogen dynamics of these geographically distinct wild populations. Future work should aim to investigate *Plasmodium* prevalence over consistent seasons between wild populations and for an extended period of time, focussing particularly on seasonal and distribution prevalence differences in free-ranging birds. Additionally, a prolonged study would allow the investigation of the long-term survival of infected birds, and may allow future pathogen load measurements and infective strain identification. The LSU-RNA qPCR used in this study offers increased potential to detect low circulating parasitaemia, and so should be considered for use in future survey work of wild birds. Furthermore, *Plasmodium* prevalence monitoring in rehabilitation birds should continue, accompanied by work to monitor the effects of vector reduction strategies and the efficacy of prophylactic treatments. Lastly, the results of this study also provide the basis for future research into the significance of the novel *Eimeria* species documented. Ongoing opportunistic monitoring is required to identify the tissue tropism of this novel protozoa, and to determine the host effects of the parasite during environmental stressors. Future molecular analysis is also required to confirm that a single species of *Eimeria* infects both distinct populations of yellow-eyed penguins.

Conservation and management implications

The aim of this research was to examine differences in host-pathogen dynamics between the geographically distinct populations of the yellow-eyed penguin, to inform predictions on the population's response to future climate shifts or stressors. The results of this study provide some insight into the possible effects of climate change and provides information to enhance the success of management strategies aimed at ensuring the survival of the yellow-eyed penguin.

Avian malaria is an emerging disease in New Zealand with a growing host range and prevalence, that will continue to increase with the effects of climate change (Schoener *et al.*, 2014, Alley and Gartrell, 2019). The results of this study suggest with reasonable confidence that the sub-Antarctic population of wild yellow-eyed penguins is free from avian malaria at the time of sampling. If these results are accurate, avian malaria may be one of the limitations to the species expanding into the northerly habitat of the New Zealand South Island. In addition, the warming temperatures in the sub-Antarctic islands (Richard *et al.*, 2013) could allow avian malaria to establish in this previously naïve ecosystem, especially if competent vectors of avian malaria increase in abundance in these areas. The loss of species and continued distribution restrictions of the remaining Hawaiian avifauna offer an example of the potentially devastating population level effects of avian malaria being introduced into this naïve population (Van Riper III *et al.*, 1986, Atkinson and LaPointe, 2009, LaPointe *et al.*, 2016). Therefore, future monitoring of avian malaria prevalence and mortality rates in wild

yellow-eyed penguins is critical to understand the emerging threat of this pathogen, and to inform future management decisions.

The results of this study also suggest that avian malaria has the potential to significantly impact the success of rehabilitation efforts of the yellow-eyed penguin in New Zealand. The growing threats to the survival of the species necessitate ongoing conservation actions including high levels of rehabilitation of young, sick or injured birds annually. The results of this study show a significantly higher prevalence of *Plasmodium* infection in rehabilitation birds, compared to wild penguins sampled. Infection of rehabilitation birds can cause direct mortality of individuals as well as significantly prolong hospitalisation periods (Parsons and Underhill, 2005, Vanstreels *et al.*, 2014, Parsons *et al.*, 2018). In addition, it is possible that birds infected in rehabilitation could become chronic carriers of the disease and introduce novel lineages of *Plasmodium* spp. into previously naïve wild populations (Brossy *et al.*, 1999, Vanstreels *et al.*, 2015, Botes *et al.*, 2017). Therefore, I suggest the use of vector exclusion measures to decrease the incidence of avian malaria in rehabilitation centres through preventing exposure of the penguins to local *Plasmodium* spp. during rehabilitation. Examples of vector exclusion methods include the use of pesticide strips, covering rehabilitation facilities in mosquito netting or housing penguins in indoor facilities, particularly during the hours of peak vector activity, to minimise vector exposure (Grilo *et al.,* 2016). Vector exclusion methods will increase the success of rehabilitation efforts by decreasing the incidence of avian malaria overall, thereby decreasing morbidity and mortality and reducing the risk of introducing novel lineages of *Plasmodium* spp. into the wild (Botes *et al.*, 2017, Vanstreels *et al.*, 2019). Preventative drugs offer an additional strategy for use in rehabilitation facilities in New Zealand. Prophylatic drug treatments are used widely in zoological facilities to reduce the morbidity and mortality associated with outbreaks of avian malaria in captive penguins (Grilo *et al.*, 2016). 8-Aminoquinolones are a commonly used class of prophylaxis as they have broad activity against the sporogonic stages developing in the mosquito host and also the asexual and sexual blood stages within penguins (Baird, 2019). Drug prophylaxis does not prevent the spread and emergence of avian malaria in facilities though, and care must be taken in the administration of the medications due to side-effects documented in penguins (Grilo *et al.*, 2016). Therefore, the judicious use of antimalarial medications could be used to complement vector exclusion methods to minimise the impact of avian malaria on the rehabilitation of yellow-eyed penguins in New Zealand.

This study has identified that there is an ongoing need for disease surveillance in the geographically distinct populations of the yellow-eyed penguin, offering results to serve as a platform for further research. The results of this study demonstrated similarities in the prevalence and pathogen load of a novel *Eimeria* between the two genetically and geographically distinct populations of yellow-eyed penguins, suggesting possible co-evolution of this host and parasite. However, the study also documented significant differences in the prevalence of avian malaria between the same populations, identifying this pathogen as a possible emerging

threat to the continued persistence of the species with the ongoing effects of climate change.

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