Investigating *Toxoplasma gondii* in the marine environment in New Zealand: from cats to kai moana (shellfish)

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Abstract

Recent reports indicate that *Toxoplasma gondii* may be an important cause of mortality for the endangered Hector's dolphin in New Zealand. Infections are thought to occur after dolphin exposure to freshwater runoff containing *T. gondii* oocysts, which are only shed by cats. This thesis investigated land-sea transmission of *T. gondii* in an effort to determine how widespread the parasite is in New Zealand coastal waters and to better understand whether representatives of variant Type II *T. gondii* are particularly pathogenic for Hector's dolphins.

Chapters 3 – 5 investigated feline infections in New Zealand, their effect on the environmental oocyst burden, and associated *T. gondii* genotypes. Chapter 3 used a novel Bayesian model to evaluate the performance of three serological assays in the absence of a gold standard, with true seroprevalence in companion cats estimated at 61 %. No significant differences were found between regions sampled, suggesting that *T. gondii* exposure is widespread, and relatively high compared to the worldwide estimate of 30 – 40 %. Chapter 4 found that 1.6 % of feral and stray cats sampled were shedding *T. gondii* oocysts. Although shedding prevalence was within expected global limits, between 51 and 62 trillion oocysts were determined to be shed into the environment annually, including in regions adjacent to Hector's dolphin habitat. Genotyping of oocysts revealed the presence of two unique recombinant strains. Chapter 5 described feline cases of toxoplasmosis in New Zealand. A rare manifestation - toxoplasmic gastritis - was uncovered, only previously reported in one other naturally infected cat overseas. Partial genotyping was possible for two of five cases, revealing what appeared to be unique, atypical strains. Although the presence of variant Type II *T. gondii* could not be ruled out due to small sample sizes and PCR amplification difficulties, results from Chapters 4 and 5 support the hypothesis that this genotype is especially pathogenic for certain endemic wildlife species, notably the Hector's dolphin. The large, estimated oocyst burden also suggests substantial exposure risks for nearshore marine mammals, especially Hector's dolphins, which are known to forage in shallow, estuarine waters.

Looking to the marine environment, Chapter 2 examined archived tissues from common, dusky, and striped dolphins for *T. gondii* presence using histological, immunohistochemical and molecular methods. None of the dolphins were found to be infected with *T. gondii*. Due to issues with sample preservation and sample size it was not possible to reach definitive conclusions, but results suggest that *T. gondii* infection and disease is more prevalent in Hector's dolphins than other cetacean species in New Zealand. Whether this is due to differences in exposure or susceptibility to toxoplasmosis still remains to be determined.

Chapters 6 – 8 focused on the use of green-lipped mussels as biosentinels, for *T. gondii* surveillance, as it has not yet been possible to molecularly confirm the presence of *T. gondii* oocysts in seawater directly. Chapter 6 provides the first report of sporulated *T. gondii* oocysts and *Giardia duodenalis* in commercial green-lipped mussels in New Zealand. Specifically, using optimised molecular methods, *G. duodenalis* assemblage B, known to be pathogenic in humans, was discovered in 1 % mussels tested. Moreover, 13 mussels (n = 104) were found to be positive for *T. gondii* DNA with a true prevalence of 16.4 % obtained via Bayesian statistics, which is relatively high compared to overseas estimates. As oocysts must sporulate outside the definitive host to become infective, the study also validated a reverse-transcriptase PCR, which confirmed the presence of a sporozoite-specific marker (SporoSAG) in four mussels. Importantly, this represented the first time that sporulated, potentially infectious, *T. gondii* oocysts were confirmed to be present in shellfish globally.

Chapter 7 aimed to find a suitably rapid, cost-effective, and analytically sensitive PCR assay for testing large quantities of wild green-lipped mussel haemolymph, in order to assess the prevalence of *T. gondii* in Hector's dolphin habitat (Chapter 8). Particularly, four different PCR assays were validated for *T. gondii* detection using oocyst spiking experiments. Results identified a real-time PCR targeting a 529-bp repetitive element (rep529) as being preferable for future mussel studies, having the lowest limit of detection (5 oocysts), good correlation between oocyst concentrations and Cq values, and acceptable efficiency. Definitive confirmation of *T. gondii* DNA via direct sequencing was shown to be required using this assay, however, as the rep529 primers cross-reacted with *Sarcocystis* spp. and *N. caninum* DNA.

Chapter 8 aimed to investigate *T. gondii* in green-lipped mussels collected from field sites located in key Māui dolphin habitats. Haemolymph samples were collected between 2014 and 2017 and stored at -80°C for 5 – 36mths. A relatively high *T. gondii* prevalence was expected: in the initial stages of the study 32/166 (19.3 %) of mussel haemolymph samples tested fresh were positive for *T. gondii* DNA. Surprisingly, no haemolymph samples tested in 2017 were positive for *T. gondii* DNA, despite the use of the validated rep529 qPCR. Although not statistically significant, results of a subsequent storage study showed that fewer stored samples tested positive compared with samples processed within 72h, suggesting that long-term storage of haemolymph could negatively impact the quality and/or yield of extractable DNA. Nevertheless, the storage study showed that *T. gondii* was still present in wild green-lipped mussels, albeit at lower prevalence (2 %), supporting previous findings that *T. gondii* oocysts are reaching coastal waters in sufficient quantities to be detected in mussels grown in New Zealand. Moreover, the results also confirmed that *T. gondii* is still present in the habitat of the critically endangered Māui dolphin subspecies and therefore remains a pathogen of concern for the Hector's dolphin.

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1 Introduction and Literature Review

1.1 Introduction

New Zealand is a global hotspot for whale and dolphin watching as over half the world's cetacean species have been reported in New Zealand's waters. Efforts to assess threats against and to conserve native cetaceans are therefore a priority in the country (Department of Conservation, 2017). Six species of dolphin are known residents but perhaps the most well-known and charismatic of these is the Hector's dolphin (*Cephalorhyncus hectori*), one of the world's smallest cetaceans, endemic to the coastal waters of New Zealand. Recent research has identified the terrestrially derived protozoan parasite, *Toxoplasma gondii*, as a pathogen of concern for the Hector's dolphin (Roe et al., 2013; Fisheries New Zealand and the Department of Conservation, 2019; Roberts et al., 2019a, b), making research into *T. gondii* in the marine environment in New Zealand an important area to pursue, to better understand epidemiology and risk factors for *T. gondii* infection and disease, as well as risk factors for marine pollution.

In fact, toxoplasmosis is a prominent emerging disease of cetaceans and other marine mammals worldwide, caused by infection with the protozoan parasite, *T. gondii*. Although parasite exposure has now been widely reported in marine mammal species, it has only been recognised as a significant cause of mortality at a population level for the southern sea otter (*Enhydra lutris nereis*) off the Californian coast, USA (Miller et al., 2002a, 2004; Kreuder et al., 2003; Bressem et al., 2009), and perhaps the Hawaiian monk seal (Barbieri et al., 2016). Toxoplasmosis, however, also appears to be a major cause of mortality for the endangered Hector's dolphin *(Cephalorhynchus hectori*) in New Zealand, including the critically endangered Māui subspecies (*Cephalorhynchus hectori maui)*. An unusually high prevalence of *T. gondii* infection and fatal toxoplasmosis in a case series of stranded or bycaught Hector's dolphins led Roe et al. (2013) to hypothesise that this parasite is either widespread in New Zealand or is particularly pathogenic for these endangered dolphins and may have population effects.

Already accepted as a serious problem for ovine livestock in New Zealand for many years (Hartley & Marshall, 1957), *T. gondii* has only recently been identified in Hector's dolphins and other endemic marine mammals (Roe et al., 2013, 2017; Michael et al., 2016). As such, little is yet known about environmental transmission and epidemiology of *T. gondii* in the marine environment in the country. Freshwater runoff contaminated with cat faeces is considered to be the source of sea otter infection in the USA (Miller et al., 2002a, 2004, 2008) and this is likely to be the case in New Zealand. Cats are the only known definitive hosts of *T. gondii* and can shed millions of environmentally robust oocysts in their faeces. Oocysts can survive for at least 24 months in saltwater, may accumulate in benthic sediment (Shapiro et al., 2012b), and can concentrate in filter-feeding invertebrates and fish, the latter being potential vectors of disease for Hector's dolphins which are documented to consume filter-feeding fish as prey (Arkush et al., 2003; Miller et al., 2008; Massie et al., 2010; Miller et al., 2013; Marino et al., 2019). Proximity of Hector's dolphins to the coastline, estuaries, and freshwater sources, as well as their preference for benthic foraging, means that exposure to oocysts reaching the sea in coastal run-off could be considerable.

A particular challenge for researchers is how to determine the prevalence of *T. gondii* in coastal habitats and risk factors for coastal contamination. A growing body of evidence suggests that filter-feeding bivalves from coastal waters can be used as bioindicators of aquatic contamination with terrestrially derived pathogens, such as *T. gondii* (e.g. Arkush et al., 2003; Palos Ladeiro et al., 2015; Shapiro et al., 2015). Importantly, the use of bivalves as biosentinels for *T. gondii* appears to be the best current option for surveillance in coastal waters, as it has not yet been possible to molecularly confirm the presence of *T. gondii* oocysts in seawater (Shapiro et al., 2019a). Contamination of shellfish with *T. gondii* has been demonstrated in a number of different countries across the globe (e.g. Esmerini et al., 2010; Putignani et al., 2011; Aksoy et al., 2014; Staggs et al., 2015), including in California mussels (*Mytilus californianus*) sampled from sea otter habitat (Miller et al., 2008; Shapiro et al., 2015). As yet, *T. gondii* has not been investigated in New Zealand shellfish, despite evidence of coastal pollution with the parasite. Green-lipped mussels (*Perna canaliculus*) are filter-feeding bivalves found all along the New Zealand coastline including in

Hector's dolphin habitat. Capable of filtering 200 L of seawater per day (James et al., 2001), this species may be a suitable bioindicator of marine environmental contamination by *T. gondii* in the country. By determining prevalence and risk factors for contamination in these mussels, exposure risks for the endangered Hector's dolphin could better be understood. Green-lipped mussels are also widely consumed by New Zealanders – gathered recreationally and farmed commercially – so surveillance for *T. gondii* and other terrestrial protozoans in this species is also relevant from a public health perspective.

Knowledge of feline infections and the environmental *T. gondii* oocyst burden in New Zealand is also essential to understand exposure risks for Hector's dolphins. This is because cats play a pivotal role in the transmission of *T. gondii* as the only definitive host and source of environmentally robust oocysts (Dubey, 2016; Di Genova et al., 2019). Indeed, evidence shows that human exposure to *T. gondii* is near or equal to zero on islands where cats have not been introduced or have been eradicated (de Wit et al., 2019). Investigation of feline infections in areas adjacent to sea otter habitat in California has proved invaluable in consolidating the land-sea transmission hypothesis, whereby oocysts shed on land are believed to make their way to the ocean in surface runoff (Miller et al., 2002). Particularly, molecular analyses have shown that the same *T. gondii* strains virulent for sea otters are found in felids sampled from sites along the sea otter range (VanWormer et al., 2014; Shapiro et al., 2019b). Feline shedding prevalence and cat densities have also been identified as important predictors of sea otter exposure (VanWormer et al., 2016; Burgess et al., 2018). Studying feline infections in New Zealand has additional importance as *T. gondii* oocysts have been identified as a source of infection for endemic wildlife (Howe et al., 2014) and livestock (West, 2002; Patel et al., 2017, 2019) and are likely to be a source of exposure for humans too (Shapiro et al., 2019a). Knowledge of feline *T. gondii* infections in New Zealand will further be vital for future risk assessments and for directing cat management strategies.

Thus, the over-arching aim of this thesis was to investigate land-sea transmission of the protozoan parasite, *T. gondii*, in New Zealand. This chapter (Chapter 1) provides a background to the lifecycle of *T. gondii*, its infectious stages, population genetics, and how it can be detected in tissues and in the environment; examines the state of knowledge of toxoplasmosis in marine mammals and reviews the literature surrounding land-sea transmission of the parasite to consider how best to study the phenomenon in New Zealand.

1.2 Literature Review

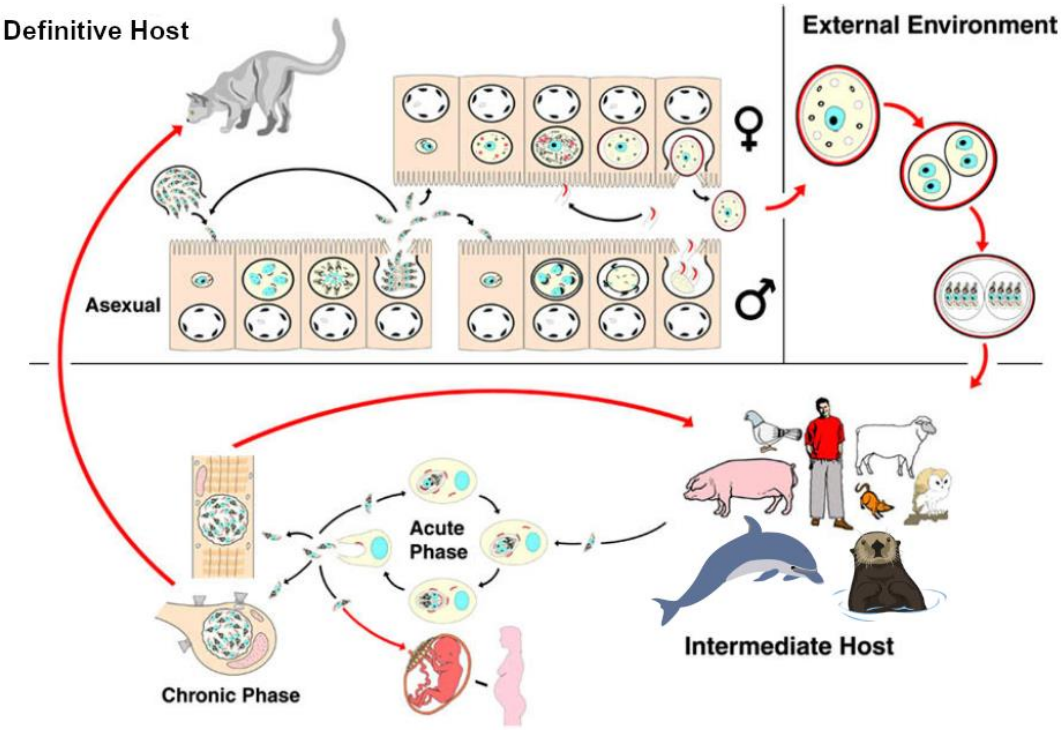
1.2.1 *Toxoplasma gondii* Lifecycle and Biology

Toxoplasma gondii is a single-celled intracellular parasite belonging to protozoan phylum Apicomplexa, subclass Coccidia, and is the causative agent of toxoplasmosis. First described over 100 years ago in the North African rodent, *Ctenodactylus gundi*, and in Brazilian rabbits (reviewed in Ferguson, 2009), *T. gondii* is now known to be one of the most successful parasites in the world, found on land and in freshwater and marine habitats. It has an extremely wide host range, is thought to be capable of infecting all warm-blooded animals, including humans, companion animals, livestock, and wildlife, and has been detected on all continents (Shapiro et al., 2010; Simon et al., 2013; Shwab et al., 2014; Dubey, 2016). *Toxoplasma gondii* has emerged as one of the most prevalent pathogens worldwide and is considered to be a parasite of both medical and veterinary importance (Dubey, 2009; Ferguson, 2009); although infections are often asymptomatic, *T. gondii* can cause serious disease and death in humans and other animals (Kreuder et al., 2003; Carme et al., 2009; Jones & Dubey, 2012).

Toxoplasma gondii is a cyst–forming coccidian, with a heteroxenous life cycle that alternates between sexual replication in the intestinal epithelium of its definitive host and asexual replication that occurs in tissues of intermediate hosts, summarised in Figure 1. There are three infectious life stages, namely

sporozoites (in oocysts), bradyzoites (in tissue cysts), and tachyzoites (in groups or clones) (Dubey et al., 1998). The lifecycle of *T. gondii* was not fully described until 1969-70, after the *T. gondii* oocyst was discovered in cat faeces, and found to be the product of sexual reproduction of the parasite in the feline intestinal epithelium (Hutchison et al., 1969, 1970, 1971; Dubey et al., 1970a, 1970b; Frenkel, 1970; Overdulve, 1970; Sheffield & Melton, 1970; Weiland & Kühn, 1970; Witte & Piekarski, 1970). To date, domestic and wild felids are the only known definitive hosts of *T. gondii* (Di Genova et al., 2019), and an individual cat can shed millions of oocysts in its faeces after infection (Frenkel et al., 1970; Dubey et al., 2009a). In contrast, all warm-blooded vertebrates, including cats, can seemingly serve as intermediate hosts of *T. gondii* in which the parasite reproduces asexually (Dubey, 2016). Tachyzoites and bradyzoites are the asexual intracellular stages of *T. gondii* found in intermediate hosts. There are three primary transmission routes for *T. gondii* which include ingestion of tissue cysts (bradyzoites) or tachyzoites in infected animal tissues, vertical transmission of tachyzoites from mother to foetus, and ingestion of oocysts (sporozoites) from contaminated water, soil, or foods (Dubey et al., 1970b; Frenkel, 1970; Tenter et al., 2000; Dubey, 2004).

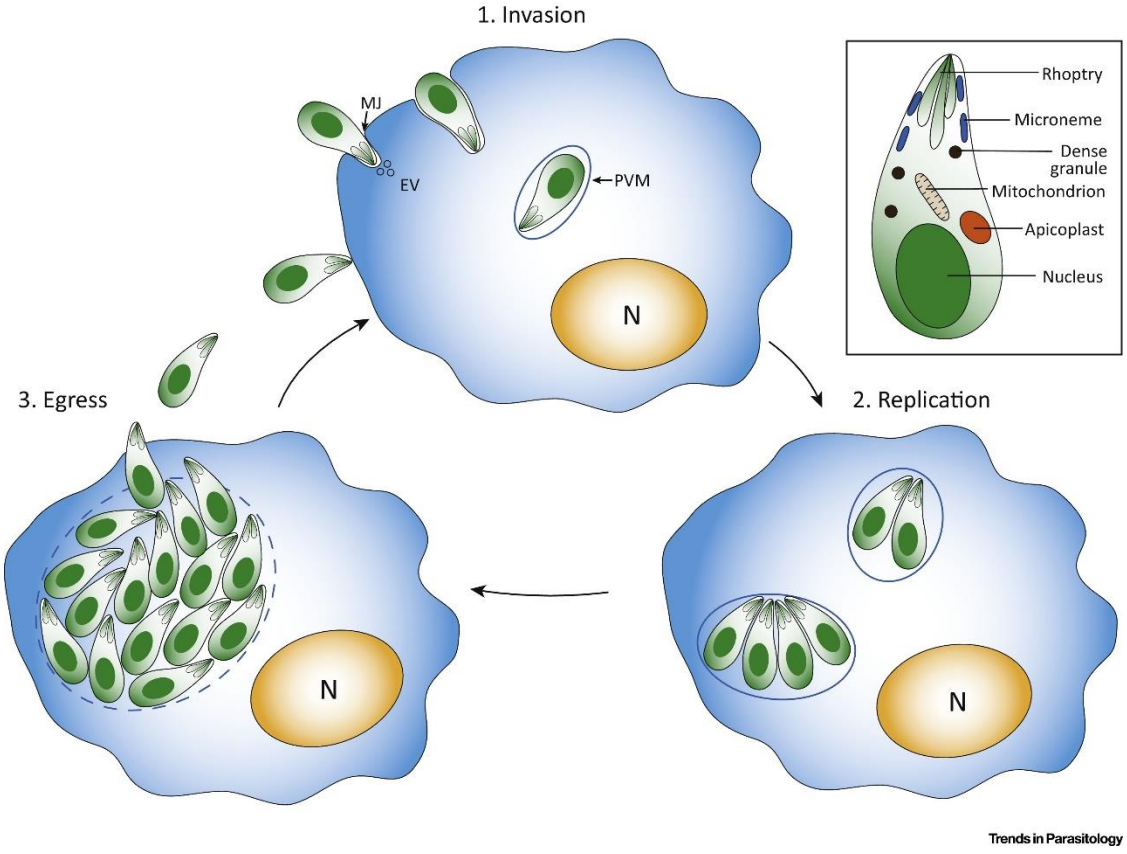
Figure 1 Diagram representing the life cycle of *Toxoplasma gondii*. The cat is the definitive host of *T. gondii* with sexual development occurring in the feline small intestine, resulting in the production, and shedding of oocysts. Oocysts contaminate the environment in cat faeces and sporulate to become infectious. Sporulated oocysts, when ingested, can infect a wide range of intermediate hosts on land and in the sea, including humans, livestock, and wildlife. Within the intermediate host, *T. gondii* undergoes asexual development. An acute phase of infection in which tachyzoites proliferate is followed by a chronic/latent phase, characterised by bradyzoites residing in tissue cysts, predominately located in neuromuscular tissue. Consumption of tissue cysts in meat can also transmit infection to other intermediate hosts and to cats. In addition, if the acute phase occurs during pregnancy, tachyzoites can cross the placenta and infect the foetus (congenital transmission). Red arrows show transmission between hosts while black arrows represent *T. gondii* development within the host. Adapted from Ferguson (2002) and Freppel et al., (2019) with permission.



1.2.1.1 Tachyzoites

When *T. gondii* is ingested by intermediate hosts, bradyzoites or sporozoites are released from cysts and invade the intestinal mucosa, where they develop into tachyzoites. Tachyzoites are often crescent-shaped, approximately 2 by 6 µm in size, with a pointed anterior end and a rounded posterior end (Figure 2; Dubey et al., 1998). *Toxoplasma gondii* tachyzoites can invade all nucleated cells of intermediate hosts and are responsible for the acute stage of infection (Figure 1; Clough & Frickel, 2017). Tachyzoites actively invade host cells, forming a parasitophorous vacuole from the host cell membrane, in which they rapidly replicate via endodyogeny (Morisaki et al., 1995; Dubey et al., 1998; Blader et al., 2015). Following replication, tachyzoites actively egress from the host cell, which is destroyed in the process. Free tachyzoites infect neighbouring cells and disseminate throughout the host, continuing the cycle of invasion, replication, and egress, which is known as the lytic cycle (Figure 2; Blader et al., 2015; Bisio & Soldati-Favre, 2019). Tachyzoites disseminate rapidly to almost all tissues, including muscle, brain, eyes, liver, lungs, and placenta (Randall & Hunter, 2011). Symptomatic infection, i.e. toxoplasmosis, results from tissue destruction due to host cell lysis and an uncontrolled inflammatory immune response (Dubey et al., 2009a; Randall & Hunter, 2011; Dupont et al., 2012; Blader et al., 2015; Harker et al., 2015). Most *T. gondii* infections, however, are regarded as asymptomatic or mild and self-resolving, as a strong interferon-gamma-dependent (IFN-γ) cell mediated immune response controls tachyzoite replication and actually kills the majority of tachyzoites during acute infection (Suzuki et al., 1988; Skariah et al., 2010; Mordue & Hunter, 2013).

Figure 2 The lytic cycle of *Toxoplasma gondii* in a host cell. The lytic cycle comprises the three main stages of invasion, replication, and egress. (1) A tachyzoite attaches to and actively invades the host cell and a parasitophorous vacuole is formed. (2) The parasite resides within the parasitophorous vacuole where it rapidly replicates by endodyogeny, protected from the host defence machinery. (3) After many rounds of replication, the parasites rupture the parasitophorous vacuole and egress into the extracellular environment. Released tachyzoites invade further host cells and continue to proliferate in the infected host. Inset: Structure of a tachyzoite showing parasite organelles. Abbreviatons: PV, parasitophorous vacuole; PVM, parasitophorous vacuolar membrane; MJ, moving junction; EV, evacuoles; N, nucleus. From Clough & Frickel (2017) with permission.



1.2.1.2 Bradyzoites

In otherwise healthy hosts, before being eliminated by the immune response, some of the remaining tachyzoites differentiate into bradyzoites. Structurally, bradyzoites differ only slightly from tachyzoites, they are crescent-shaped, but more slender measuring approximately 7 by 1.5 µm, having a nucleus situated towards the posterior, whilst it is more central in tachyzoites (Dubey et al., 1998). Bradyzoites also reside in an intracellular parasitophorous vacuole, which matures into a thick-walled tissue cyst (Dubey et al., 1998; Knoll et al., 2013). Tissue cysts vary in size from 5 to 100 µm and contain a few to several hundred bradyzoites (Dubey et al., 1998). Although tissue cysts may develop in visceral organs, including lungs, liver, and kidneys, they are more prevalent in muscular and neural tissues, including the brain, eye, skeletal, and cardiac muscle (Dubey et al., 1998; Miller et al., 2018). Although tissue tropism in *T. gondii* infection is not fully understood, it is thought that most hosts remain infected for life and have persistent tissue cysts (Dubey, 2016). At this stage, infection is called chronic or latent. As bradyzoites contained within cysts are considered to be outside of host immune defenses, latent infection is not usually considered to be associated with any overt symptoms. It was previously thought that bradyzoites were non-replicative or slow growing (Dubey et al., 1998), but recent research suggests that they are more dynamic, and can show episodic growth and replication (Watts et al., 2015; Sinai et al., 2017). The dynamics and processes involved are not fully understood but findings show that tissue cysts may rupture sporadically releasing bradyzoites; infection is kept in check by host immunity (Mordue & Hunter, 2013; Dubey, 2016). Latent infection can therefore be a serious problem for hosts that become immunocompromised, bradyzoites then convert back to the invasive and destructive tachyzoite form. This is known as recrudescence of acute infection.

1.2.1.3 Oocysts

Oocysts of *T. gondii* are produced after sexual replication of the parasite in the definitive host, a felid. Ingestion of any of the three infectious stages of *T. gondii* by a cat can cause oocyst production and shedding, but the process of sexual replication is best documented in cats that have ingested tissue cysts

(Dubey et al., 1998). In this case, after ingestion, digestive enzymes break down cyst walls, releasing bradyzoites into the stomach and small intestine. Bradyzoites penetrate epithelial cells of the small intestine where they undergo five stages of asexual development before male and female gamonts are formed and fertilisation occurs to form oocysts (see Fig. 1 in Dubey & Frenkel, 1972). Oocysts are discharged into the lumen of the cat gut as infected intestinal epithelial cells rupture and are subsequently passed into the environment in faeces. As such, oocysts are the only environmental form of *T. gondii*, which otherwise is an obligate intracellular parasite.

Clinically normal cats can defecate millions of *T. gondii* oocysts (Miller et al., 2018). Experimental and natural infections of domestic cats (*Felis catus*) have shown that the quantity of oocysts shed per infection varies between 3 and 810 million, occasionally none (Dubey, 1976, 2001, 2002, 2005; Schares et al., 2008b; Dabritz & Conrad, 2010). The shedding period lasts for a median of 8 days but may be as long as three weeks (Dubey, 1976, 2001, 2002, 2005). The time to oocyst shedding after infection (pre-patent period) reportedly varies depending on the stage of *T. gondii* ingested by the cat (Dubey & Frenkel, 1972, 1976; Freyre et al., 1989; Dubey, 1996, 1998a; Dubey et al., 1998). Pre-patent periods are three to ten days after ingesting tissue cysts, ≥13 days after ingesting tachyzoites, and ≥18 days after ingesting oocysts. The frequency of oocyst shedding has also been found to depend on the infectious stage ingested (Dubey & Frenkel 1976; Dubey, 1996); less than 30 % of cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingesting tissue cysts. In nature, cats can be infected through ingestion of any of the three infectious stages of *T. gondii,* but domestic cats are mainly thought to become infected with *T. gondii* after consuming tissue cysts in prey, such as small mammals and birds (Davis & Dubey, 1995; Dubey, 2016; Simon et al., 2018). There has also been growing interest in feline immunity and number of times a cat will shed oocysts in its lifetime, as this affects the environmental burden (Zulpo et al., 2018). As yet, no vaccines are approved for veterinary use to prevent oocyst excretion following feline infection (Ramakrishnan et al., 2019).

Oocysts are shed in faeces in an unsporulated state and must sporulate to become infectious. Sporulation occurs outside the cat within one to five days of oocyst excretion and is dependent on environmental conditions such as aeration and temperature (Dubey et al., 1970b; Frenkel, 1970). Whilst unsporulated oocysts are subspherical to spherical, 10 by 12 µm in diameter, sporulated oocysts are subspherical to ellipsoidal, 11 by 13 µm in diameter. Each sporulated oocyst contains two ellipsoidal sporocysts, measuring 6 by 8 µm, and each sporocyst contains four sporozoites (Dubey et al., 1998). Interestingly, *T. gondii* oocysts autofluoresce under UV excitation (330-385 nm) (Lindquist et al., 2003).

1.2.1.3.1 Environmental resistance of *T. gondii* oocysts and infectious dose

Oocysts are known to be highly resistant in both soil and water and to most chemical and physical disinfectants; resistance is attributed to structural, molecular, and biophysical properties of both oocyst and sporocyst walls (see Freppel et al., 2019; Shapiro et al., 2019a). Experimental studies have shown that sporulated oocysts remain infective for up to 18 months in soil depending on humidity, temperature and exposure to direct sunlight (Yilmaz & Hopkins, 1972; Frenkel et al., 1975; Lélu et al., 2012), and for six up to 54 months in freshwater (Dubey, 1998b). Multiple experiments have shown that common freshwater treatment methods, including chlorination, application of iodine tablets, ozonification, UV irradiation, and radio frequency heating, are ineffective in completely inactivating *T. gondii* oocysts (Wainwright et al., 2007a, b; Dumètre et al., 2008). Furthermore, although *T. gondii* oocysts are reportedly inactivated by exposure to ≥60 °C for 1 min, higher temperature and longer duration may be necessary for complete and reliable inactivation (VanWormer et al., 2013a; Travaillé et al., 2016). Of particular interest is the ability of oocysts to sporulate and retain infectivity when exposed to seawater. Lindsay and co-authors (2003) tested this ability and found that 75 – 80 % of oocysts kept in 15 and 32 ppt artificial sea water sporulated within three days and were still infectious for mice after six months storage in 15 ppt seawater at 4°C or room temperature (Lindsay et al., 2003). Survival over the longer-term was also investigated by Lindsay & Dubey (2009) who demonstrated that sporulated *T. gondii* oocysts can survive in seawater for at least 24 months. As Rousseau et al. (2018) note, however, these studies were terminated while at least some of the oocysts

were still infective, meaning the outer limit of viability and infectivity for *T. gondii* oocysts deposited in seawater is not yet known. Nevertheless, the ability of *T. gondii* oocysts to survive in seawater for prolonged periods greatly facilitates dissemination through different waterway environments (Shapiro et al., 2019). For example, oocysts shed by felines and carried into freshwater rivers could remain viable when dispersed into estuarine or seawater locations. Environmental resistance likely enhances the probability of transmission to marine species and broadens the range of available hosts.

The number of oocysts necessary to infect intermediate and definitive hosts appears to vary with the host species, but experimental studies have shown that a single *T. gondii* oocyst can be infective to susceptible pigs, rats, or mice (Dubey, 1996; Dubey et al., 1996; Dubey, 1997). Ingestion of 100 oocysts can result in infection and oocyst shedding in cats and result in further contamination of the environment with oocysts (Dubey, 1996). For *T. gondii*, the ID_{50} (number of oocysts required to infect 50% of exposed subjects) is estimated between one and 10 oocysts in rodent models (Dubey, 2016), and ingestion of a single oocyst can infect susceptible swine (Dubey et al., 1996). The low infectious dose for *T. gondii* means that the associated risk to animal and human health is potentially high.

1.2.2 Toxoplasmosis in humans and other animals

For humans with a healthy immune system, *T. gondii* infections are usually asymptomatic or mild/subclinical with flu-like symptoms, for reasons detailed above. Although approximately 30 % of the entire human population is estimated to be infected with *T. gondii* (Tenter et al., 2000; Innes, 2010), clinical toxoplasmosis cases are generally limited to immunologically impaired individuals and vertically infected foetuses or newborns. Acute or recrudescent infections in HIV patients and patients receiving immunosuppressive therapy can have serious consequences, resulting in meningoencephalitis and death (Dubey & Jones, 2008; Pereira-Chioccola et al., 2009). In pregnant women, initial infection with *T. gondii* can result in abortion, and congenital toxoplasmosis can result in permanent neurological disabilities and

severe visual impairment in newborns, from birth or developing during childhood (McLeod et al., 2006; Olariu et al., 2011; Bahia-Oliveira et al., 2017).

Whilst these at-risk groups are more likely to be clinically affected by *T. gondii* infection, it is now known that immunocompetent individuals can develop clinical disease and even fatal systemic toxoplasmosis: ocular disease (toxoplasmic choroiditis) is a fairly common consequence of *T. gondii* infection despite immunocompetence (Dubey & Jones, 2008; Anand et al., 2012; Arantes et al., 2015; Guo et al., 2015) and severe and fatal disease has been documented in healthy individuals (e.g. Demar et al., 2012). Outbreaks of toxoplasmosis have also been documented, predominantly in countries outside of Europe and North America, which are often linked to food or waterborne transmission of *T. gondii* oocysts (Benenson et al., 1982; Bowie et al., 1997; Tenter et al., 2000; Dubey, 2004; Conrad et al., 2005; Shapiro et al., 2019a).

Interestingly, the recent development of a serologic test to distinguish oocyst- from tissue cyst-acquired infections revealed that the ingestion of oocysts is likely to be a more important source of infection for humans than ingestion of tissue cysts in undercooked meat (Boyer et al., 2011; Hill et al., 2011). It has also recently been questioned whether latent *T. gondii* infections are actually asymptomatic, as they have been associated with chronic neurological and psychiatric conditions, particularly schizophrenia and bipolar disorder (Fabiani et al., 2015; Ngoungou et al., 2015; Fuglewicz et al., 2017). Although causal links have yet to be established, it is worth noting that there are no drugs or treatments available to cure chronic *T. gondii* infection (Innes et al., 2019). It is unknown to what extent the severity of toxoplasmosis in humans depends on host genetics, parasite-host interactions, the life-stage of *T. gondii* causing infection, parasite genotype, or other factors (Dubey & Jones, 2008).

In general, animal infections with *T. gondii* follow a similar path to those in humans, with widespread exposure to the parasite documented in many species across the globe, but clinical toxoplasmosis generally

limited to at-risk populations (Dubey, 2016). Some species, however, do appear to be more susceptible to disease processes after infection. For example, infection is well-documented to impact negatively on reproductive success in sheep (Hartley & Marshall, 1957; Dubey & Jones, 2008; Dubey, 2016), goats (Dubey & Jones, 2008), and deer (Patel et al., 2019). Initial infection with *T. gondii* during pregnancy can result in abortion storms and further research (although limited) suggests that chronic infection can lead to recurrent reproductive problems in sheep (Hide et al., 2009) and perhaps in other animals such as rodents (Rejmanek et al., 2010), dogs (Bresciani et al., 2009), and sea otters (Shapiro et al., 2016). Australian marsupials are thought to be highly susceptible to *T. gondii* infection, with a number of fatal cases reported (Donahoe et al., 2015; Dubey, 2016). Similarly, *T. gondii* appears to be particularly pathogenic for some marine mammals, notably Southern sea otters (Kreuder et al., 2003), and perhaps Hector's dolphins (Roe et al., 2013) and Hawaiian monk seals (Barbieri et al., 2016). In contrast, pigs rarely develop clinical toxoplasmosis, and no confirmed clinical cases have yet been reported in horses or cattle (Dubey, 2016). As for humans, reasons for susceptibility and disease are complex and not fully understood. Potential risk factors for individuals, groups, or species, include age, immune status, recent introduction of cats, stage of *T. gondii* ingested, genotype of infecting strain, diet, habitat use and anthropogenic development, amongst others.

In New Zealand, *T. gondii* has long been considered a problem for livestock. In fact, the veterinary importance of *T. gondii* worldwide was first established when the parasite was found to be the cause of abortion in New Zealand sheep flocks, leading to large economic losses (Hartley & Marshall, 1957). Currently, exposure to *T. gondii* is associated with suboptimal reproductive performance in sheep and farmed deer in the country (West, 2002; Patel et al., 2012, 2017, 2019). Infection with *T. gondii* may also impact the human population. Although available data is sparse, infection appears to be prevalent with a seroprevalence of 43 % found amongst blood donors (Zarkovic et al., 2007), and may be an under-reported problem in pregnant women (Moor et al., 2000). In addition, fatal and systemic cases of toxoplasmosis, as well latent *T. gondii* infections, have now been reported in native wildlife, including bird species vulnerable

or at risk of extinction (Howe et al., 2014) and endangered marine mammals (Roe et al., 2013, 2017). Of particular concern is the recent discovery that toxoplasmosis may be playing a role in the population decline, or failure to recover, of the Hector's dolphin, including the critically endangered Māui dolphin subspecies (Roe et al., 2013; Roberts et al., 2019a, b). The species of livestock and native wildlife found to be affected by toxoplasmosis in New Zealand so far are not likely to consume tissue cysts in infected warm-blooded prey, and whilst transplacental transmission certainly occurs in some of these species, findings suggest that environmental transmission and oocyst-acquired infections may be especially important in this country.

1.2.3 Genotypes of *T. gondii*

Although *T. gondii* is considered a single species within the genus *Toxoplasma*, nearly 200 different genotypes of the parasite have been described based on the alleles present at multiple polymorphic genetic loci in *T. gondii* strains (Howe & Sibley, 1995; Su et al., 2012; Shwab et al., 2014). For epidemiological studies and to study the transmission routes of the parasite it is important to identify and compare genotypes of *T. gondii* in definitive and intermediate hosts (Miller et al., 2008; VanWormer et al., 2014; Shapiro et al., 2019a, 2019b). Genotyping is also necessary because the infecting *T. gondii* genotype may be associated with disease severity (Grigg et al., 2001; Saeij et al., 2005; Shapiro et al., 2019b). Over the last two decades several genotyping markers have been identified which permit researchers to distinguish between *T. gondii* genotypes using PCR-based methods (Su et al., 2010, 2012; Dardé et al., 2013). The most commonly encountered methods in the literature involve the use of multilocus restriction fragment length polymorphism (RFLP) markers and microsatellite (MS) markers, as well as multi-locus sequence typing (MLST) (reviewed in Dardé et al., 2013; Liu et al., 2015).

This review focuses on PCR-RFLP and also discusses direct sequencing of typing loci. This is because the PCR-RFLP technique developed by Su and colleagues (2006, 2010) is still favoured to genotype *T. gondii*, and

is often augmented with sequence typing to provide finer distinction between strains (e.g. Miller et al., 2008; Sundar et al., 2008; Donahoe et al., 2014, 2015; VanWormer et al., 2014; Shapiro et al., 2015, 2019b; Brennan et al., 2016). PCR-RFLP is also the current method of choice to genotype *T. gondii* in New Zealand (Roe et al., 2013, 2017; Howe et al., 2014; Mirza et al., 2017; Patel et al., 2019) and using different methods to analyse strains complicates comparison (Su et al., 2012).

Typing of *T. gondii* genetic loci using PCR-RFLP is based on the ability of endonucleases to recognise single nucleotide polymorphisms (SNPs) in DNA sequences, to digest PCR products, and subsequently show distinct DNA banding patterns by agarose gel electrophoresis (Howe & Sibley, 1995; Su et al., 2010; Dardé et al., 2013). Traditionally, the method is based on the analysis of ten markers distributed over eight chromosomes and one apicoplastic marker, namely SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico (Su et al., 2010). In contrast, sequence analysis involves direct sequencing of PCR products from typing loci and comparison of sequence data with those of reference isolates, looking for characteristic and novel DNA sequence polymorphisms including SNPs, nucleotide insertion and deletion (e.g. Miller et al., 2008; Sundar et al., 2008; Pan et al., 2012; Brennan et al., 2016; Shapiro et al 2019b). Virtual RFLP can also be carried out, whereby computer simulations of endonuclease activity (e.g. NEBCut-ter (New England Biolabs Inc.; http://tools.neb.com/NEBcutter2/index.php)) are used on sequence data from PCR products of typing loci (Donahoe et al., 2014, 2015; Brennan et al., 2016; Shapiro et al., 2019b). For direct sequencing analyses, the typing markers of Su et al. (2006) are often used along with the B1 gene, which is also known to be polymorphic (Grigg & Boothroyd, 2001).

Early studies using PCR-RFLP revealed the dominance of three clonal strains of *T. gondii* amongst human and animal isolates from North America and Europe, which were named Type I, Type II, and Type III (Sibley & Boothroyd, 1992; Howe & Sibley, 1995). As such, the population structure of *T. gondii* was thought to be fairly simple with limited genetic diversity. Since then, *T. gondii* strains from a large number of hosts -

humans, domestic and wild animals - from across the globe, have been characterised and compared, revealing a much more complex population structure (Su et al., 2012; Jiang et al., 2018; Shwab et al., 2014, 2018). Although the use of different typing methods and markers has complicated strain comparison (Su et al., 2012), population genetic analyses have confirmed the existence of the three archetypal genotypes of *T. gondii* (Types I – III) as well as the existence of numerous recombinant and atypical strains (summarised in Dardé et al., 2013). Recombinant genotypes, derived from crosses between the archetypal lineages, have a mix of archetypal alleles at polymorphic loci, whilst atypical strains are those which have novel alleles (Grigg et al., 2001; Pan et al., 2012). Hosts can also have mixed infections, meaning they are infected with multiple *T. gondii* genotypes, with two alleles observed concurrently at one or more loci, or different genotypes identified in different tissues sampled from the same host (Pan et al., 2012).

The use of different methods and markers has generated significant data to aid our understanding of *T. gondii* genetic diversity and population genetics. However, given that each method has its own scheme of classification, genotype designation has been confusing (Dardé et al., 2013). A standardised nomenclature is offered by the Toxoplasma genome database (ToxoDB, http://www.toxodb.org/toxo/, Gajria et al., 2007), which gives a number to strains based on multilocus PCR-RFLP typing results: Type I (ToxoDB #10), II (ToxoDB #1, #3), III (ToxoDB #2), noting that Type II *T. gondii* is now considered to be made up of clonal Type II (ToxoDB #1) as well as variant Type II (ToxoDB #3), which only differ at the Apico locus (Dardé et al., 2013). Variant Type II is also sometimes referred to as atypical Type II in the literature (e.g. Roe et al., 2013; Cooper et al., 2016). Dardé et al. (2013) provide a useful comparison of designations for over a hundred *T. gondii* genotypes.

Through population genetic analyses, distinct geographical patterns in *T. gondii* genotypes have emerged (Su et al., 2012; Shwab et al., 2014). As summarised by Galal et al. (2019), in Europe, Type II (ToxoDB #1, #3), and to a lesser extent, Type III (ToxoDB #2) strains predominate in domestic and wild environments. In

North America, Type II (ToxoDB #1, #3) and Type III (ToxoDB #2) genotypes are also prevalent, particularly in humans and domestic animals. However, atypical genotypes belonging to Type 12 (ToxoDB #4 and #5), notably Type X and Type A (ToxoDB #5) predominate in wildlife, although they are also found in domestic animals, attributed to their encroachment on home ranges of wild felids (Su et al., 2006; Miller et al., 2008; Wendte et al., 2011; VanWormer et al., 2014). Less research has been carried out on strains from Africa and Asia, but so far Type III (ToxoDB #2) and variant Type II (ToxoDB #3) appear to dominate in Africa, while genotypes Chinese 1(ToxoDB #9) and Type 1(ToxoDB #10) are prevalent in Asia (Wendte et al., 2011; Shwab et al., 2014; Chaichan et al., 2017). In contrast, despite substantial research efforts, no dominant strains have been identified in South America where a significant proportion of atypical strains are found, and genetic diversity of *T. gondii* is particularly high both in domestic and wild environments (Ajzenberg et al., 2005; Khan et al., 2005; Lehmann et al., 2006; Pena et al., 2008; Shwab et al., 2014, 2018).

In Australia, genotyping data is limited, but Type II-representative strains (#1- and #3-like) are reportedly most common, with classic Type II RFLP banding patterns across most or all loci examined, but with novel alleles identified through sequence analysis, notably at the B1 locus (Donahoe et al., 2014; Cooper et al., 2015; Donahoe et al., 2015; Brennan et al., 2016; Cooper et al., 2016). Findings have led authors to purport that Type II-like strains with unique B1 polymorphisms are native to and circulating in Australia, identified in a Risso's dolphin (*Grampus griseus*), a New Zealand fur seal (*Arctocephalus forsteri*), a wallaby (*Macropus rufogriseus*), three wombats (*Vombatus ursinus*), a peach-faced love bird (*Agapornis roseicollis*), a human, and eight domestic cats (Sibley & Boothroyd, 1992; Al-Qassab et al., 2009; Parameswaran et al., 2010; Donahoe et al., 2014, 2015; Shwab et al., 2014; Cooper et al., 2015, 2016; Donahoe et al., 2015; Brennan et al., 2016). Recombinant and other atypical strains, as well as mixed infections, however, also seem to be prevalent in Australian wildlife (Parameswaran et al., 2010; Pan et al., 2012). Although further data is needed, identifying the same genotype in animals, humans, and cats suggests that Type II-like strains are widespread in Australia. Whether or not these genotypes are particularly pathogenic for Australian wildlife remains to be determined, as only a limited number of clinically diseased and healthy animals from wild and

domestic environments have been tested and compared (Parameswaran et al., 2010; Pan et al., 2012). Results from Australia, however, also indicate that the use of sequence analysis, rather than reliance on PCR-RFLP, increases the likelihood of finding atypical alleles and greater genetic diversity, as noted previously by Miller et al. (2004), Sundar et al. (2008), amongst others.

Genotyping data from New Zealand is particularly limited. To date, multilocus PCR-RLFP (without sequence analysis) results have only been published from wildlife cases. Fatal cases of toxoplasmosis in Hector's dolphins and neuromuscular toxoplasmosis in a New Zealand sea lion were attributed to variant Type II (ToxoDB #3), or variant Type II-like *T. gondii* strains (Roe et al., 2013, 2017). Likewise, fatal toxoplasmosis in endemic wild birds was linked to variant Type II *T. gondii*, which led Roe et al. (2013, 2017) to hypothesise that this genotype is either widespread or particularly pathogenic for New Zealand wildlife. Since then, studies have shown that more diverse strains are circulating in New Zealand. Specifically, Mirza et al. (2017) genotyped *T. gondii* strains from latently infected wild raptors that had no histological lesions of toxoplasmosis but were *T. gondii* positive on PCR. Although genotyping was not successful at all typing loci, the same unique recombinant genotype pattern was identified in all eight isolates from six birds, showing a mix of Type I (ToxoDB #10) and Type II (ToxoDB #1, #3) alleles. Findings suggest that representatives of the variant Type II lineage may indeed be particularly pathogenic for New Zealand wildlife. However, whether this is the case would require genotyping of more isolates from clinically diseased versus healthy wild and domestic animals, including cats. The inclusion of cats could also shed light on land-sea transmission of the parasite (discussed below).

Despite the observed regional specificity in genotypes, and higher prevalence of atypical genotypes in wildlife in some locales, PCR-RFLP genotypes #1 (Type II clonal), #2 (Type III), #3 (Type II variant) and #10 (Type I) are identified globally (Shwab et al., 2014; Jiang et al., 2018). Furthermore, genotypes in the Type II lineage, ToxoDB #1 (clonal Type II) and ToxoDB #3 (variant Type II), appear to be the most common in the

world to date, found in cats, humans, livestock, and wildlife, both on land and in the sea (Sibley & Boothroyd, 1992; Dubey et al., 2009b, 2011; Miller et al., 2008; Sundar et al., 2008; Al-Qassab et al., 2009; Al-Kappany et al., 2010; Parameswaran et al., 2010; Roe et al., 2013, 2017; Howe et al., 2014; Donahoe et al., 2014, 2015; Shwab et al., 2014; VanWormer et al., 2014; Cooper et al., 2015, 2016; Brennan et al., 2016).

1.2.4 Detection of *T. gondii* and diagnosis of toxoplasmosis or *T. gondii* infection

Understanding land-sea transmission of *T. gondii*, with the ultimate aim of managing toxoplasmosis in a marine mammal species, requires both the successful diagnosis of toxoplasmosis or *T. gondii* infection in key hosts and detection of environmental contamination with *T. gondii* oocysts. Genetic characterisation of *T. gondii* strains found along the land-sea gradient is also crucial for surveillance and epidemiological study of toxoplasmosis in the marine environment (Miller et al., 2008; VanWormer et al., 2013a). It is therefore pertinent to review the most important techniques used for detection and diagnosis, recognising that a large body of literature is dedicated to this topic, so this review cannot be all-encompassing. There are many methods available for the detection of *T. gondii* in biological and environmental samples including microscopy, bioassay, a variety of serological assays, and molecular methods based on detection of *T. gondii* nucleic acids, particularly polymerase chain reaction (PCR). The history and development of diagnostic tests has been reviewed elsewhere (e.g. Liu et al., 2015; Wyrosdick & Schaefer, 2015). Several methods for genotyping are also available, as discussed previously. Generally, a combination of tests is required to confirm the presence of the parasite, particularly as parasite numbers may be low (Su et al., 2010).

Following Montoya (2002) and Miller et al. (2018), in this thesis the term 'toxoplasmosis' is reserved to describe the clinical or pathological disease caused by *T. gondii*, and '*T. gondii* infection' describes an asymptomatic primary infection or persistence of the parasite in tissues (chronic or latent infection).

Clinical signs of toxoplasmosis are not pathognomonic in humans or animals. A diagnosis of *T. gondii* infection or toxoplasmosis is therefore usually established by a combination of laboratory tests. Diagnostic methods include serologic assays, histologic demonstration of the parasite and/or its antigens (i.e. immunohistochemistry, IHC), isolation of the parasite using animal bioassay or cell culture, and amplification of specific nucleic acid sequences of *T. gondii* (i.e. PCR) (Montoya, 2002). This review focuses on diagnostic methods used in the veterinary field. When evaluating diagnostic tests, research has shown that it is important to consider their accuracy, meaning it is important to estimate the sensitivity and specificity of each test. Diagnostic sensitivity refers to the ability of an assay to correctly identify true positive samples, defined as the proportion of samples that test positive out of the total number of true positives. Diagnostic specificity refers to the ability of an assay to correctly identify true negative samples, defined as the proportion of samples that test negative out of the total number of true negative samples (Dohoo et al., 2013). Knowledge and comparison of these test characteristics is not always considered in the published literature but is recommended; test characteristics not only affect the optimal choice of test/s to be used in the population under investigation but will also affect prevalence estimates (Johnson et al., 2019).

1.2.4.1 Serology

Serologic tests are used for diagnostic identification of *T. gondii* infection using a host's serum and are based on the immune response of the host after exposure to the parasite, i.e. antibody-antigen reactions. After a host is infected, the innate immune response limits parasite growth and promotes the development of adaptive immunity which is required for long term resistance to infection (Hunter & Sibley, 2012). The adaptive immune response includes the production of *T. gondii*-specific antibodies within days to weeks of primary infection (Sabin & Feldman, 1948; Lappin & Powell, 1991; Wastling et al., 1995), notably immunoglobin M (IgM) and immunoglobin G (IgG). Specific *T. gondii* IgG antibodies usually remain detectable for years (Montoya, 2002). Serological tests are one of the primary means of testing humans

(Montoya, 2002) and are also commonly used in the veterinary world. They are generally used in live populations but can also be used postmortem (Dubey et al., 2003; Miller et al., 2004).

A variety of serological tests are used to detect serum antibodies against *T. gondii* infection, including agglutination tests (latex agglutination test (LAT), direct agglutination test (DAT), modified agglutination test (MAT)), fluorescence-based tests (e.g. indirect fluorescent antibody test (IFAT)), Western blot (WB), and enzyme-linked immunosorbent assays (ELISA)) (Desmonts & Remington, 1980; Dubey & Desmonts, 1987; Dubey & Thulliez, 1989; Sohn & Nam, 1999; Miller et al., 2002; Silva et al., 2002; Dabritz et al., 2007; Wyrosdick & Schaefer, 2015) . According to Wyrosdick and Schaefer (2015), agglutination testing remains the most commonly used modality for the diagnosis of toxoplasmosis in live animals. This is because these tests do not rely on the use of a species-specific protein conjugate, unlike ELISAs and immunofluorescence tests. Of these, the MAT is preferred (Dubey, 2016), perhaps because it has been found to be more sensitive in some species (Dubey & Thulliez, 1989).

Serological tests are useful because they can be used to determine prevalence of *T. gondii* infection in a population. For the majority of hosts, infections are considered to be largely asymptomatic and resolve with minimal or no pathology, but the immune system fails to achieve a sterile immunity and a stable persistent infection results (Mordue & Hunter, 2013). Therefore, an individual shown to be exposed to the parasite (seropositive), is considered to be infected (Lappin, 2010). Sero-surveys therefore permit analysis of differences in *T. gondii* infection levels in and between populations and geographical locations (e.g. Miller et al., 2002a, b), and investigation of other risk factors associated with infection (e.g. Dabritz et al., 2007; Opsteegh et al., 2012, discussed in more detail below).

Despite this, there are limitations associated with serological testing, particularly the requirement for host species-specific conjugates (ELISA, IFAT and WB) and a lack of availability of commercial tests (Wyrosdick &

Schaefer, 2015; Patel et al., 2017). Furthermore, variable performance of different tests in different hosts means that the evaluation of test characteristics – diagnostic sensitivity (Se) and specificity (Sp) – is a necessary prerequisite for interpretation of test results in sero-surveys (Joseph et al., 1995; Greiner & Gardner, 2000; Mainar-Jaime & Barberán, 2007; Pan-ngum et al., 2013; Johnson et al., 2019). Test evaluation ideally should be carried out in the species and population in which the test is to be implemented (Greiner & Gardner, 2000; Miller et al 2002a; Dabritz et al., 2007; Mainar-Jaime & Barberán, 2007). Evaluation, however, can be difficult to carry out if a gold standard test is not available and/or known positive samples are not available, but can be achieved using statistical modelling, such as Bayesian latent class analysis (Patel et al., 2017; Johnson et al., 2019). Furthermore, seroprevalence does not provide information about the severity or genotype of infection, and serological tests cannot be used to test environmental samples.

1.2.4.2 Necropsy, Histology, and Immunohistochemistry (IHC)

Necropsy examinations are vital for diagnosis of toxoplasmosis as a primary or contributing cause of death and are therefore particularly important for examining marine mammals, which in many locations are only examined after death when they strand and are recovered. At necropsy, gross lesions of toxoplasmosis are often absent or non-specific. Diagnosis of active or fatal infection can be achieved by detecting typical histological lesions of toxoplasmosis, although these are not pathognomonic of the disease, and can vary with species and route of transmission. A definitive diagnosis therefore requires demonstration of protozoal organisms within histological lesions in host tissue, followed by confirmation of *T. gondii* using immunohistochemistry (IHC), and PCR or electron microscopy. Immunohistochemical analysis involves using commercially produced anti-*T. gondii* antibodies to check for certain *T. gondii* antigens in a sample of tissue. The antibodies are linked to an enzyme or a fluorescent dye. After the antibodies bind to the antigen in the tissue sample, the enzyme or dye is activated, and the antigen, i.e. *T. gondii* organisms, can then be seen under a microscope (Buchwalow & Böcker, 2010). For *T. gondii*, the dominant antigenic epitopes expressed by tachyzoites continue to be expressed by bradyzoites. As a result, IHC using

polyclonal antisera raised against tachyzoites is an effective means for detection of tachyzoites, bradyzoites and tissue cyst walls in infected tissues (Miller et al., 2009).

Necropsy, histology, and immunohistochemistry are vital for postmortem diagnosis of toxoplasmosis, but recent research has stressed the importance of tissue selection (Elmore et al., 2016). *Toxoplasma gondii* tachyzoites are capable of penetrating any nucleated cell and, in cases of disseminated toxoplasmosis, many organs are affected. However, *T. gondii* infection is usually not fatal and is often chronic with tachyzoite conversion to bradyzoites within tissue cysts. Although results of some studies suggest that *T. gondii* cysts are consistently found in the brain and heart of infected animals, *T. gondii* infection may exhibit tissue tropism as well as uneven distribution of the parasite within organs, dependent on host species, *T. gondii* strain, *T. gondii* life stage causing infection, and inoculation route (Dubey, 1997; Dubey et al., 2003; Elmore et al., 2016). For these reasons, histology and immunohistochemistry are of little use in detecting latent infections. For example, Dubey et al. (2003) report that in naturally exposed pigs, the density of *T. gondii* may be as low as 1 tissue cyst in 100 g of tissue, which is not likely to be detected by histology, by bioassay in mice, or by cell culture.

1.2.4.3 Transmission electron microscopy

Transmission electron microscopy (TEM) can be used for identification of *T. gondii* organisms through observation of distinctive ultrastructural features, which are not evident using light microscopy due to the small size of the parasite. In TEM, a beam of electrons is shone through an ultrathin section of tissue to observe features at very high resolution. The use of electrons to illuminate the sample rather than visible light enables features to be viewed at far greater resolution than light microscopy meaning TEM is particularly useful for determining how components inside a cell, such as organelles, are structured. TEM has been used to characterise the ultrastructure of *T. gondii* organisms, and the electron microscopic data on *T. gondii* and its life cycle stages is reviewed in Dubey et al. (1998), Ferguson & Dubremetz (2013). The

features which distinguish *T. gondii* organisms from other morphologically similar protozoans, namely *Hammondia* spp., *Sarcocystis* spp., *Neospora* spp., are described in Speer et al. (1999) and Dubey & Sreekumar (2003).

TEM has previously been used by researchers investigating toxoplasmosis in marine wildlife and in cats to demonstrate the presence of *T. gondii* tachyzoites associated with lesions in tissue sections, confirming the aetiological agent in cases of fatal or active toxoplasmosis (e.g. Inskeep et al., 1990; Bowater et al., 2003; Ploeg et al., 2011; Bossart et al., 2012; Gonzales-Viera et al., 2013; Tommasi et al., 2014). Inskeep et al. (1990) provided the first report of toxoplasmosis in cetaceans published in the English language, with the discovery of fatal disseminated disease in a female Atlantic bottlenose dolphin (*Tursiops truncatus*) that stranded in Florida, USA. Their diagnosis was aided by ultrastructural confirmation of numerous intralesional *T. gondii* tachyzoites and a tissue cyst in the brain. Tommasi et al. (2014) described an unusual case of biliary coccidiosis caused by *T. gondii* in a domestic cat which was euthanised due to a history of severe hepatic failure. The authors performed TEM to show intracellular tachyzoites in the cytoplasm of biliary cells. However, in this study, molecular methods were considered requisite for definitive confirmation that *T. gondii* organisms were present.

It should be noted, however, that TEM is not commonly utilised in postmortem studies of toxoplasmosis and, since the advent of PCR, confirmation of the presence of *T. gondii* is preferentially carried out using molecular methods (e.g. Cole et al., 2000; Miller et al., 2004; Roe et al., 2013), even when TEM is included (e.g. Tommasi et al., 2014). This is likely because PCR is relatively simple and sensitive, and analysis of sequencing results can quickly differentiate between closely related protozoans. In contrast, electron microscopy requires a skilled microscopist, special tissue preparation, and is relatively time-consuming and expensive. It may also prove difficult to ultrastructurally distinguish between *T. gondii* organisms and morphologically similar protozoans (Tommasi et al., 2014). TEM may therefore be particularly useful where

PCR is not available or where PCR results are not definitive (see Chapter 5). Also, whilst PCR can generally only confirm the presence of *T. gondii* DNA/RNA, TEM can give a detailed visualisation of the life stage of *T. gondii* in the specimen, which may help clarify whether an infection is active or latent (Bossart et al., 2012; Tommasi et al., 2014), and whether an active infection is primary or recrudescent (Bowater et al., 2003). Overall, TEM is an important adjunct technique in unusual cases/presentations of toxoplasmosis (e.g. Gonzales-Viera et al., 2013) but is not suitable for routine testing.

1.2.4.4 Polymerase chain reaction (PCR) for detection and characterisation of *T. gondii*

Molecular methods use polymerase chain reaction (PCR) to amplify *T. gondii*-specific DNA or RNA in biological and environmental samples (Su et al., 2010). Polymerase chain reaction is a technique in which a specific target sequence of DNA/RNA in a template is amplified using complementary primers and polymerase enzymes to produce millions of copies of the target. There are several types of PCR used for detection of *T. gondii* DNA/RNA, notably conventional PCR, nested PCR (nPCR), and real-time PCR (qPCR). Reverse-transcriptase PCR (RT-PCR) is used when the starting template in the reaction is RNA, and the RNA is first transcribed to its DNA complement by reverse transcriptase enzymes. RT-PCR may make use of standard (conventional, nested), or real-time technologies (e.g. Miller et al., 2008; Villegas et al., 2010). In standard PCR and RT-PCR (conventional/nested), PCR products, i.e. amplicons, are visualised by gel electrophoresis. Relative band sizes and intensities can be used to determine specific amplification and to obtain a rough estimate of target expression levels. Amplicons can also be sequenced and compared to reference isolates to confirm the presence of the target.

In qPCR amplification is visualised in real-time, and a positive reaction is detected by accumulation of a fluorescent signal generated by an intercalating dye, such as SYBR Green, or from the breakdown of a dye-labeled probe during amplification of the target sequence. This may be used to detect the presence or absence of target nucleic acids (qualitative qPCR), or their amount (quantitative qPCR). The PCR cycle

where the fluorescent signal first becomes discernible above background noise is called the C_T (cycle threshold) value (Stephenson, 2016). C_T levels are inversely proportional to the amount of target nucleic acid in the samples, i.e. the lower the C_T level, the greater the amount of target nucleic acid in the sample. Of note, accurate primer design and optimisation of reaction conditions is important when using intercalating dyes like SYBR Green since the dye will bind to all double stranded DNA molecules, potentially resulting in non-specific amplification and/or primer dimers, which form when two primers bind to one another instead of to the template DNA, due to regions of primer complementarity (Best & Roberts, 2014). Probe-based technologies are commonly used for *T. gondii* detection, e.g. TaqMan or FAM-based probes (e.g. Miller et al., 2002a; Arkush et al., 2003; Kasper et al., 2009). These technologies are considered to be more specific than intercalating dyes as they require specific hybridisation between the probes and target DNA sequence before fluorescence is emitted. Probe-based assays are therefore preferred in the *T. gondii* literature but are expensive. SYBR Green and other intercalating dyes are also used (e.g. Edvinsson et al., 2006; Aigner et al., 2010; Marques et al., 2020), however, as alternatives to probe-based methods as they are simple to use, reliable, and cheap, thus a practical tool for detecting *T. gondii*, especially when dealing with a large number of samples.

Important issues to consider with PCR are the analytical sensitivity and specificity of assays. In contrast to diagnostic sensitivity and specificity, discussed above, analytical sensitivity refers to the minimum number of copies in a sample that can be measured accurately with an assay. Typically, analytical sensitivity is expressed as the limit of detection (LOD), which is the concentration that can be detected with reasonable certainty with a given analytical procedure. Analytical sensitivity can be defined as the lowest concentration of the parasite that can be consistently detected amongst replicates. Analytical specificity refers to the PCR assay detecting the appropriate target sequence rather than other, nonspecific targets also present in a sample (Bustin et al., 2009).

Since the advent of PCR in the 1980s, molecular methods have become fundamental tools for the detection and genetic characterisation of *T. gondii* in biological samples. Initially developed for use in human medicine (Burg et al., 1989), PCR-based methods now have extensive application and have been used to detect *T. gondii* in a wide range of tissues from humans and other animals. These include fresh, frozen, and formalin-fixed paraffin embedded (FFPE) tissues, as well as environmental samples such as cat faeces, soil, or water, and bioindicator samples such as shellfish and other paratenic hosts (e.g. Arkush et al., 2003; Lélu et al., 2012; Shapiro et al., 2010, 2015; Krusor et al., 2015). PCR-based assays are particularly important for testing environmental samples as examination for *T. gondii* oocysts by microscopy alone, even if a concentration step is employed, may not be definitive (Hohweyer et al., 2013). Elements approximately 11 by 13 µm in size with sporocyst-like structures containing crescent-shaped sporozoites that autofluoresce blue when excited to 300 – 385 nm may indicate the presence of sporulated oocysts of *T. gondii*, but oocysts of *Hammondia* spp. and *Neospora* spp. also exhibit the same size, structure, and autofluorescence pattern (Lindquist et al., 2003). The use of bioassays for confirmatory purposes with environmental samples is also challenging (see section 1.2.5.3.2 Determining infectivity and viability of *T. gondii* oocysts in shellfish, pg. 63).

PCR was first used to detect *T. gondii* DNA by Burg et al. (1989), who successfully developed a conventional PCR to amplify the B1 gene of *T. gondii*. The assay was able to detect as few as 10 parasites in 100,000 human leukocytes. Since then, many other markers in the *T. gondii* genome have been used to indicate the presence of the parasite in biological samples and to characterise strain types, reviewed by Su et al. (2010) and Dardé et al. (2013). The number of parasites in biological samples tend to be low (Su et al., 2010). Therefore, if only identification of *T. gondii* is required, small repetitive DNA sequences are preferable as amplification targets. This is because small sequences are amplified more efficiently than large ones and because there are more template copies in a repetitive sequence per organism, thereby increasing the analytical sensitivity that can be achieved. This means assays targeting multi-copy markers should have higher analytical sensitivities than assays targeting single copy markers.

Three such genetic markers are often used to detect *T. gondii*, namely the 35-copy B1 gene (Burg et al., 1989), the 110-copy internal transcribed spacer (ITS-1) or 18S rDNA gene sequences (Hurtado et al., 2001; Jauregui et al., 2001; Calderaro et al., 2006), and the 200 – 300 copy 529 bp repeat element (rep529) (Homan et al., 2000). As highlighted by Dardé et al. (2013), since their initial development, methods using these markers have been modified and adapted for use with different PCR technologies – conventional, nested, or real-time PCR – in many different laboratories worldwide. At the time this project began, the most sensitive assay reported was the rep529 quantitative real-time PCR assay of Kasper et al. (2009), capable of detecting 1/50 of a genome equivalent of *T. gondii* (Kasper et al., 2009; Su et al., 2010), and rep529-based qPCR assays have been correlated with higher sensitivity for parasite detection in clinical (Homan et al., 2000; Edvinsson et al., 2006), food (Hohweyer et al., 2016), and water (Yang et al., 2009) samples. An alternative genetic marker is the *dhps* gene of *T. gondii* (Aspinall et al., 2002b). In New Zealand, a nested PCR (nPCR) amplifying a small fragment of the *dhps* gene is preferentially used for the detection of *T. gondii* DNA and molecular confirmation of *T. gondii* infection. Although this marker has successfully been utilised to confirm the presence of *T. gondii* in Hector's dolphins (Roe et al., 2013), a fur seal, (Roe et al., 2017), native birds (Howe et al., 2014; Mirza et al., 2017), and farmed deer (Patel et al., 2019), the number of copies of the *dhps* gene in the *T. gondii* genome is not yet known, and this assay has not been validated to determine its analytical sensitivity and specificity.

Overall, the main issues highlighted in the literature are that method standardisation for *T. gondii* detection is lacking, making inter-study comparisons difficult (Su et al., 2010; Dardé et al., 2013; Shapiro et al., 2015). There is an increasing focus on trying to develop the most analytically sensitive test, however, recent research also highlights the need to ensure analytical specificity of the target, especially when dealing with environmental samples (Shapiro et al., 2015; Bahia-Oliveira et al., 2017; Shapiro et al 2019a). Of note, whether a one-size-fits-all standardised method for all sample types is possible is not known, and assays ideally should be validated for use with the intended matrices. It should also be noted that isolation of *T. gondii* from wildlife is known to be time consuming, expensive, and difficult. The quality of DNA from

naturally infected wildlife is often poor as most tissues are often collected long after death. The density of *T. gondii* in tissues of asymptomatic animals is also low making it challenging to draw comparative conclusions about determinants of disease (Dubey et al., 2011).

With respect to strain characterisation, genotyping of *T. gondii* traditionally relies on single copy markers that are polymorphic between strains, such as the surface antigen genes SAG1, SAG2, SAG3, and others in Su et al. (2010) used in multilocus PCR-RFLP (see section 1.2.3 Genotypes of *T. gondii*, pg. 15). Unfortunately, although the rep529 marker of *T. gondii* appears to offer the greatest analytical sensitivity for PCR-based detection of the parasite, it cannot be used for genotyping as nucleotide sequences of this repetitive element are highly conserved among different strains, isolates, and among different intragenomic copies (Reischl et al., 2003). With respect to the *dhps* gene, sequencing has shown that polymorphisms are present but that they can only distinguish Type I (ToxoDB #10) *T. gondii* from other genotypes (Pashley et al., 1997; Aspinall et al., 2002a; Meneceur et al., 2008). The polymorphisms found in the *dhps* gene, however, are not captured by the marker of Aspinall et al. (2002b) used in New Zealand.

Reliance on the single-copy markers of Su et al. (2010) for genotyping *T. gondii* in biological, and particularly environmental samples may be challenging, however (Esmerini et al., 2010; Shapiro et al., 2015; Staggs et al., 2015), as discussed in section 1.2.5.3.1 Challenges facing *T. gondii* detection in shellfish, pg. 61. These assays usually require a relatively large amount of parasite DNA in a sample for successful amplification (Khan et al., 2005) and RFLP methods were generally developed using DNA from isolates rather than tissue/environmental samples (VanWormer et al., 2014). Due to these issues, some authors have adopted (and adapted) the B1 marker of Grigg & Boothroyd (2001) for both detection and genotyping purposes (e.g. Miller et al., 2004, 2008; Costa et al., 2011, 2013; Donahoe et al., 2014, 2015; VanWormer et al., 2014; Shapiro et al., 2015; Brennan et al., 2016; Shapiro et al., 2019b). This marker is both multicopy and polymorphic, amplifying restriction sites needed for RFLP.

These authors have also moved towards direct sequencing of genotyping markers, including the B1 gene. This approach is preferred because mutations may not affect restriction enzyme digestion outcomes, thus PCR-RFLP genotyping alone is unable to fully assess the true extent of polymorphism between alleles (Donahoe et al., 2014). However, it is also important to be able to compare results between studies and in New Zealand, previous research has utilised traditional PCR-RFLP (e.g. Roe et al., 2013; Howe et al., 2014). Furthermore, direct sequencing is also relatively costly, which may be prohibitive in large studies.

In summary, serological testing can be used to assess exposure risk and infection prevalence in intermediate and definitive hosts. Necropsy is also required for a definitive diagnosis of toxoplasmosis as a cause or contributing cause of death, which requires finding *T. gondii* organisms associated with lesions in host tissues. This can be achieved using microscopy of tissue sections subject to hematoxylin and eosin (H&E) staining, and IHC, but further testing, e.g. parasite isolation through bioassay or cell culture, transmission electron microscopy, extraction of parasite DNA/RNA and PCR, genotyping and sequencing of isolates (see Miller et al., 2008, Dubey et al., 2009a, and Dardé et al., 2013 for more information) is usually considered necessary for confirmation, particularly as *T. gondii* is morphologically similar to other protozoan parasites and must be differentiated from *Sarcocystis* species and *Neospora caninum* (Dubey et al., 1998). Necropsy and light microscopy are poor tools for detecting latent infections, due to tissue tropism of *T. gondii* and low parasite numbers in latent infections (Dubey et al., 1997; Elmore et al., 2016). The use of multiple techniques aids diagnosis of the full spectrum of *T. gondii* infections, including fatal disseminated disease, active toxoplasmosis, and latent infection, in animals of interest.

For environmental and bioindicator samples, PCR-based methods are the primary means of *T. gondii* oocyst detection; recent reviews (Bahia-Oliveira et al., 2017; Shapiro et al., 2019a) highlight, however, that sequence analysis is important for definitive confirmation following PCR due to the potential for non-target amplification by flora and biota commonly present in both gut and environmental habitats – even when

using primers shown to be specific for *T. gondii* when compared with other protozoan parasites (Shapiro et al., 2015) or enteric pathogens (Lilly & Wortham, 2013). Animal bioassays or other molecular methods can also be used with environmental and bioindicator samples to determine parasite viability. Microscopy is available but oocysts of *T. gondii* may be indistinguishable from those of closely related protozoans.

1.2.5 Studying land-sea transmission of *T. gondii*

1.2.5.1 *Toxoplasma gondii* in marine mammals

In the 1970s, the first cases of toxoplasmosis in marine mammals were reported, in pinnipeds (Pelt & Dieterich, 1973; Migaki et al., 1977; Holshuh et al., 1985; Miller et al., 2001), then cetaceans (Bandoli & de Oliveira, 1977; Cruickshank et al., 1990; Inskeep et al., 1990; Migaki et al., 1990; Mikaelian et al., 2000; Resendes et al., 2002), sirenians (Buergelt & Bonde, 1983), and mustelids, notably the Southern sea otter (Cole et al., 2000). Reports of toxoplasmosis and *T. gondii* infection in many marine mammal species across the globe have now been published and *T. gondii* is considered a pathogen of concern for these animals (Miller et al., 2018).

Early marine mammal reports questioned how the affected animals might have been exposed to *T. gondii*, as few marine mammals consume warm-blooded intermediate hosts, limiting their exposure to tissue cysts. For example, Inskeep et al. (1990) described the live-stranding and subsequent death of a female Atlantic bottlenose dolphin and her calf, with *T. gondii* organisms observed in tissues of both animals, and a diagnosis of fatal disseminated toxoplasmosis reached for the adult female. The authors suggested that transplacental transmission, a common cause of clinical toxoplasmosis for many animals, including humans, could be a route of infection for cetaceans. Significantly, they also speculated that sand and coastal water could be contaminated with feline faeces containing *T. gondii* oocysts, suggesting that floodwater spillage and sewage could serve as a source of infection. Moreover, they speculated that fish, a common dietary component of dolphins could serve as intermediate/transport hosts. At the time, survival of *T. gondii*

oocysts in saltwater had not been determined, although oocysts were known to be highly resistant and terrestrial paratenic hosts had been identified experimentally.

Much of what we now know about transmission of toxoplasmosis to marine mammals comes from efforts to understand how, what is essentially a terrestrial parasite, came to infect and cause serious disease in a high proportion of Southern sea otters in California (Kreuder et al., 2003). As such, this research provides important information and lessons for future studies on *T. gondii* in the marine environment. Piecing together this puzzle, which is still underway, is very much an interdisciplinary effort. Efforts have involved marine mammal pathology, tracking, live-sampling, and modelling (Miller et al., 2002a, b, 2004, 2008; Kreuder et al., 2003; Conrad et al., 2005, 2013; Sundar et al., 2008; Johnson et al., 2009; Shapiro et al., 2012a, 2016; Burgess et al., 2013, 2018; ; VanWormer et al., 2016; Tinker et al., 2019), testing sea otter invertebrate prey as a potential source of *T. gondii* (Arkush et al., 2003; Krusor et al., 2015; Shapiro et al., 2015; Staggs et al., 2015), investigation of *T. gondii* infections and oocyst shedding in cats living on land adjacent to otter habitats (Dabritz et al., 2006, 2007a, b; Dabritz & Conrad, 2010; VanWormer et al., 2013a, b, 2014), and identifying how best to detect *T. gondii* in marine waters, whether through testing seawater directly (Shapiro et al., 2010), or by using shellfish as biosentinels (Miller et al., 2008; Shapiro et al., 2015, 2019b; Staggs et al., 2015). This section focuses on the steps taken to investigate *T. gondii* in sea otters, as well as on the findings, with a discussion on their relevance for New Zealand research. Later sections go on to discuss *T. gondii* in felids and in marine invertebrates, as prey or as biosentinels.

1.2.5.1.1 Investigating prevalence of *T. gondii* infection and mortality in the sea otter

The Southern sea otter is a federally listed threatened subspecies, only found in coastal waters off California, USA. Since the year 2000 there has been a focused effort to study the impact of *T. gondii* infection on the Southern sea otter population in coastal California (Cole et al., 2000) and to apply this knowledge to better understand the transmission of pathogenic protozoa at the human–domestic animal–

wildlife interface (Conrad et al., 2005, 2013). Research found that exposure to *T. gondii* was widespread in the Southern sea otter and that toxoplasmosis was a significant cause of death in the population. Specifically, Miller et al. (2002b) and Conrad et al. (2005) conducted serologic screening of live Southern sea otters, finding that 38 % (*n* = 257) of the otters were seropositive for *T. gondii*. Furthermore, the authors discovered that 52 % of fresh dead otters necropsied between 1998 and 2004 tested positive for anti-*T. gondii* antibodies. Further work by Kreuder et al. (2003) using the same necropsy data (1998 – 2001) discovered that toxoplasmosis was the primary cause of mortality for 16.2 % (17/105) of the otters examined and *T. gondii* infection was a contributing cause of death for a further 11.4 % (12/105) across all age classes, making it one of the two leading causes of mortality in the study population. Interestingly, otters with *T. gondii* encephalitis were more likely to be attacked and killed by sharks or die from cardiac disease, other major causes of mortality, meaning that concurrent *T. gondii* infection could play a critical role in a high level of mortality in sea otters. Given that these animals rarely consume recognised intermediate hosts of *T. gondii*, findings suggested that significant environmental exposure was occurring via water contaminated with oocysts shed in the faeces of infected cats. Efforts were therefore made to better understand how the otters were getting infected with *T. gondii* as well as factors leading to the death of such a large proportion of the otters after infection.

1.2.5.1.2 Identifying risk factors for *T. gondii* exposure and infection in the sea otter

A number of studies examined risk factors associated with *T. gondii* exposure in the Southern sea otter by combining seroprevalence, observational, demographic, and coastal environmental data (Miller et al., 2002a, b; Conrad et al., 2005; Johnson et al., 2009; Burgess et al., 2013, 2018; VanWormer et al., 2016; Tinker et al., 2019). Several factors were identified that explained variation in sea otter infection risk, including individual-level intrinsic and behavioural features of the otter, as well as features of the terrestrial and nearshore environment.

1.2.5.1.2.1 Individual-level intrinsic and behavioural features of the sea otter as risk factors

With respect to individual-level intrinsic and behavioural features, sex, diet, and age were determined to be a significant risk factors for parasite exposure by several authors (Miller et al., 2002a; Conrad et al., 2005; Johnson et al., 2009; Burgess et al., 2013, 2018; Tinker et al., 2019). As increasing age was associated with *T. gondii* seropositivity, most sea otter infections were inferred to be acquired horizontally, a finding also observed in other marine mammal species, such as sea lions (*Zalophus californianus*) (Carlson-Bremer et al., 2015). Serological data therefore supported the hypothesis that the primary route of infection with *T. gondii* for the sea otter was the ingestion of oocysts (Miller et al 2002a; Conrad et al., 2005). Findings prompted the evaluation of major otter prey species, such as mussels, oysters, and marine snails, as transport hosts of *T. gondii* oocysts (Kvitek et al., 1988; Lindsay et al., 2001, 2004; Arkush et al., 2003; Krusor et al., 2015; Shapiro et al., 2015, discussed in detail in section 1.2.5.3 Shellfish as sentinels of *T. gondii* in the marine environment, pg. 54), as well as the examination of dietary preferences as a risk factor for *T. gondii* exposure. Diet was of particular interest because sea otters in central California were found to have very high degrees of individual dietary specialisation, believed to be an adaptive mechanism for coping with limited food resources (Tinker et al., 2007; Johnson et al., 2009). Interestingly, using observational studies of live sea otters, combined with extensive health monitoring, Johnson et al. (2009), Burgess et al. (2013) and Tinker et al. (2019) discovered that marine snail consumption was associated with a higher risk of infection with *T. gondii*. Burgess et al. (2013) and Tinker et al. (2019) determined that otters that were marine snail specialists were 18.1 (95 % CI: 1.81–180.48) times more likely to be seropositive for *T. gondii* than otters specialising in other prey types. In Johnson et al. (2009), individual diet specialisation was a significant predictor of seroprevalence to the extent that, amongst otters foraging in the same site of San Simeon and Cambria, individuals preying on abalone had a 22 % probability of infection with *T. gondii*, compared with 72 % for otters with a primarily non-abalone diet, and 95 % for otters consuming marine snails. Results strongly suggested that differential infection risks existed within a locality, depending on dietary preferences.

1.2.5.1.2.2 Features of the terrestrial and nearshore environment as risk factors for sea otter exposure

Beyond this, specific high-risk locations for Southern sea otter infection have been identified along the California coast (Miller et al., 2002a; Conrad et al., 2005; Johnson et al., 2009; Burgess et al., 2013; Tinker et al., 2019). Several features of the terrestrial and nearshore environment appear to affect *T. gondii* exposure risk for the sea otter through their effect on the distribution and concentration of oocysts in otter habitat. These include proximity to freshwater outflow, domestic cat density, human population density, anthropogenic development, and land use (Miller et al 2002a; Van Wormer et al., 2016; Burgess et al., 2013, 2018; Tinker et al., 2019).

The relationship between *T. gondii* serological status and sample location was evaluated by Miller et al. (2002a), Conrad et al. (2005), Johnson et al. (2009), Burgess et al. (2013), and Tinker et al. (2019). In all studies, hotspots of *T. gondii* seropositivity were documented, with significant differences in *T. gondii* exposure evident in otters sampled from different areas within the Southern sea otter range. Both Miller et al. (2002a) and Conrad et al. (2005) detected high-risk sites around Elkhorn Slough and around Estero/Morro Bay, with Conrad et al. (2005) determining that otters from the Elkhorn Slough area were six times as likely (95 % CI: 1.9 – 20.0), and otters from San Simeon to Morro Bay were five times as likely (95 % CI: 1.9 – 16.2), to have been exposed to *T. gondii* than otters from the more remote and rocky Big Sur coast. In contrast, Burgess et al. (2013) and Tinker et al. (2019) discovered a higher seroprevalence amongst female otters in Big Sur (18.5 %), compared with those sampled in Monterey, a relatively urbanised area.

The relationship between location and sea otter infection has proved to be difficult to untangle. Spatial risk may partly be explained by differences in freshwater outflow levels between locations. In Miller et al. (2002a), most (89 %) of the 19 otters sampled in the high-risk vicinity of Elkhorn Slough were exposed to maximal freshwater flow, and otter proximity to coastal freshwater outflow was found to be strongly

associated with a higher risk of *T. gondii* infection across all study animals. The odds of seropositivity were approximately three times higher in areas of maximal outflow relative to areas with low outflow. According to the authors, in California, surface water runoff is conducted to coastal streams, or directly to the ocean from lawns, streets and open land via storm drains, ditches and culvert pipes, with essentially no pre-treatment. Environmentally resistant *T. gondii* oocysts present in cat faeces could therefore efficiently be transported to the nearshore marine environment by surface runoff. Otters living in or near large plumes of contaminated freshwater would therefore be at increased risk for *T. gondii* exposure. Coastal freshwater outflow, as calculated in this study, was considered to be roughly analogous to maximal terrestrial surface water runoff. Results therefore provided strong evidence to support a land-based origin of infection, and a significant role for freshwater runoff in the transmission of *T. gondii* to otters.

Spatial risk may also be explained by features of the terrestrial environment, namely domestic cat densities, human population densities, anthropogenic development, and land use (VanWormer et al., 2016; Burgess et al., 2018). By linking spatial oocyst loading and transport models, VanWormer et al (2016) discovered that areas of increased otter *T. gondii* seroprevalence were closely aligned to watersheds with the highest levels of oocyst runoff, which were characterised by coastal development, higher precipitation, and larger domestic cat populations. Similarly, using logistic regression analyses, Burgess et al. (2018) found high *T. gondii* prevalence to be associated with higher human population density and a greater proportion of human-dominated land uses. Findings were attributed to the notion that increased human population density and developed land uses are both associated with increased domestic cat density, thus increased oocyst loading in nearshore environments. Anthropogenic development of land may also accelerate overland flow of contaminated runoff and reduce the filtering capacity of wetlands.

However, these factors may not be sufficient to explain variations in spatial risk along the otter range. As mentioned above, Burgess et al. (2013) and Tinker et al. (2019) found a higher prevalence of infection in otters sampled in Big Sur compared with Monterey. This was unexpected as the Monterey area is relatively urbanised, with a higher density of domestic cats compared to Big Sur. The Monterey area also has many storm drains that drain urban areas with impervious surfaces, and major rivers that drain large agriculturally dominated watersheds. Big Sur, however, is considered a pristine environment, with few domestic cats and many small un-populated or sparsely populated watersheds. It instead has a higher density of bobcats and mountain lions that are also hosts of *T. gondii* and could be an important source of infectious oocysts in more pristine areas. Furthermore, although Big Sur has small, sparsely populated watersheds, the terrain is very steep, meaning that oocysts deposited on land may be quickly washed into the sea after rain events. The authors also explained that kelp beds – home of both otters and marine snails – grow very close to the shore in Big Sur. As such, otters in this area may have a higher risk of exposure, despite the relative lack of cats and freshwater sources, pointing to a need to consider local bathymetry and topography as well as host-specific factors.

Similarly, there was no clear relationship between freshwater exposure and seropositivity established in the high-risk San Simeon to Morro Bay region identified in Miller et al. (2002a) and Johnson et al. (2009), discussed above. Documented to have a fairly low human density and no substantial freshwater input, authors concluded that un-recognised factors contributed to the increased risk for *T. gondii* exposure in otters sampled from this area. Coastal geography, winds, tides, marine currents, and sedimentation were proposed to play a role in locally concentrating oocysts in the nearshore environment, influencing the distribution of oocysts in seawater and sea otter prey.

1.2.5.1.2.3 Dominant risk factors for *T. gondii* exposure depend on spatial scales

Of particular interest, the work of Burgess et al. (2018) showed that the dominant risk factors for otter exposure and infection depend on the spatial scale of analysis. Specifically, the authors collated *T. gondii* seroprevalence data encompassing the entire sea otter range from Alaska to California, including both Northern (*Enhydra lutris kenyoni*) and Southern sea otter subspecies, along with data on individual-level and terrestrial risk factors. This dataset was analysed over three spatial scales: 1) a conventional within-region analysis, limited to the study sites of Miller et al. (2002a), Conrad et al. (2005), Johnson et al. (2009), and Burgess et al. (2013); 2) an among-region analysis that compared regional average values of terrestrial risk factors to mean regional *T. gondii* prevalence, and; 3) a large-scale 'landscape' analysis incorporating the whole otter range, which used a mixed effects multivariate logistic model to estimate the significance of terrestrial variables for individual otter infection, as well as individual-level variables, including a random effect to account for dependence among samples collected in the same study region. Results showed that at smaller spatial scales (within-region), observed patterns in risk variability were better explained by individual-level risk factors, such as diet, and likely local topography, bathymetry, and ocean processes. However, at the wider scale, site-based differences in human population density, and land use, also appeared to significantly affect the distribution of infection risk. As such, the spatial level of analysis should be considered when evaluating prior research and designing new studies.

1.2.5.1.3 Identifying risk factors for sea otter mortality from toxoplasmosis

Studies have also investigated factors associated with mortality due to toxoplasmosis in the Southern sea otter, using extensive necropsy data to try and understand why the disease is a major cause of death for the subspecies. Despite researching toxoplasmosis as a cause of death for several years (Kreuder et al., 2003; Miller et al., 2004; Conrad et al., 2005; Sundar et al., 2008; Shapiro et al., 2012a), significant associations between mortality and *T. gondii* genotype, season, and year of sampling, have only recently been identified

(Shapiro et al., 2019b), highlighting the complex epidemiology of *T. gondii* infection in otters and marine wildlife in general.

It is possible that high mortality of sea otters due to toxoplasmosis can be accounted for by increased exposure of this subspecies to *T. gondii* relative to other marine mammals in California, as otters spend the majority of their lives within 500 m of the coastline, where oocyst concentrations are likely to be highest (VanWormer et al., 2013b). Indeed, Kreuder et al. (2003) discovered a potential spatial cluster of mortality in Estero Bay, in which half of the otters (8/16) recovered were determined to have *T. gondii* encephalitis as the primary cause of death. Other evidence for increased exposure driving mortality rates in the sea otter comes from research conducted on the California sea lion (*Zalophus californianus*). Although California sea lions are sympatric with the sea otter, *T. gondii* antibody prevalence was found to be substantially lower in this species, over a similar time period and using the same serologic test (Carlson-Bremer et al., 2015). Authors concluded that this may represent variable exposure to infectious oocysts – otters and sea lions have overlapping ranges yet have different feeding ecologies and migratory patterns, which likely results in different patterns of exposure within the same ecosystem.

Nevertheless, the relationship between infection and mortality has not proven to be clear cut. Most recently, Shapiro et al. (2019b) found season and year of stranding to be associated with *T. gondii* as a primary cause of death for sea otters, using multivariate logistic regression to examine the relationship between *T. gondii* genotype and death due to toxoplasmosis (discussed below). The authors discovered that sea otters stranding in the wet season were 10 times (95 % CI: 1.4 – 73.0) more likely to have toxoplasmosis as the primary cause of death than those stranding during the dry season. However, no seasonal peaks were identified in earlier studies by Kreuder et al. (2003) and Shapiro et al. (2012a). Shapiro et al. (2012a) used precipitation and stream flow data as indicators of land-based run-off to evaluate temporal associations between protozoal mortalities and runoff events in the two months preceding

death/stranding at Monterey and Estero Bay study sites (as the suspected incubation time of *T. gondii* is 7 – 30d post-exposure). However, no association between land-based runoff and sea otter deaths attributable to *T. gondii* infection was observed. Possible reasons for the lack of association in the earlier studies beyond study design factors include the complex pathophysiology of *T. gondii* in otters, where death might not occur within a short time after exposure to the parasite. Although acute toxoplasmosis is documented, infection can be latent and later recrudesce, obfuscating the relationship between time of infection and mortality. Of note, the dietary preferences of otters with toxoplasmosis as primary or contributing cause of death have not been evaluated as a risk factor for mortality, likely due to the difficulties in determining diet postmortem. Furthermore, unlike Shapiro et al. (2019b), earlier studies did not include *T. gondii* genotype in statistical models (see below), which may have confounded the relationship between season and mortality.

High levels of mortality due to toxoplasmosis in sea otters in particular areas and within the population as a whole could also be linked to other factors such as increased susceptibility of the Southern sea otter subspecies relative to other marine mammals, or enhanced parasite virulence. Several factors were proposed by Kreuder et al. (2003) and Miller et al. (2004) to contribute to a fatal outcome following *T. gondii* infection, including immunosuppression through concurrent disease, environmental pollution exposure, or inbreeding depression (see also Gibson et al., 2011). Miller et al. (2004) also proposed *T. gondii* strain variation or novel host-parasite interactions to be behind increased sea otter susceptibility, on the grounds that although *T. gondii* is a single species, significant intraspecific differences with respect to disease presentation exist. Linkages between *T. gondii* genotype and virulence have been identified in domestic animals and humans. For example, strains with predominantly Type I alleles rather than Type II or III are highly virulent for laboratory mice (Dardé, 2008). In humans, predictors of disease severity are less apparent, but more virulent toxoplasmosis has been associated with Type I strains, as well as atypical genotypes of *T. gondii* in a number of studies (Sibley & Boothroyd, 1992b; Dardé et al., 1998; Grigg et al., 2001; Demar et al., 2007; Carme et al., 2009). This line of thinking has proved to be of particular

importance as evidenced by the recent work of Shapiro et al. (2019b) who published the first study to show strain-specific impacts of *T. gondii* on otter survival.

In the study of Shapiro et al. (2019b), stranded sea otter carcasses from 1998 – 2015 were necropsied to determine primary and contributing causes of death. A total of 116 otters had both detailed necropsy and molecular data available. All 12 otters with toxoplasmosis as the primary cause of death were infected with strains classified within the Type X (ToxoDB #5) genotype. The odds of dying with toxoplasmosis as the primary cause of death were 29 times higher (95 % CI: 1.4 – 620.4) for sea otters infected with a Type X strain than those infected with Type II or a mixed Type II/X genotype. This observed association between infection with strains that possess predominantly Type X alleles and fatal toxoplasmosis in sea otters is strongly suggestive that *T. gondii* strain is an important determinant of outcome following exposure for these animals. This research also permits comparison of *T. gondii* genotypes from sea otters with strains from felids inhabiting land adjacent to otter habitat, to assess land-sea transmission and to provide additional insight on sources of infection (discussed in section 1.2.5.2 Cats and *T. gondii* transmission, pg. 44).

1.2.5.1.4 Relevance of sea otter studies for New Zealand research on Hector's dolphins

The sea otter studies discussed thus far have been paramount to understanding *T. gondii* in the marine environment in California and provide a wealth of information and guidance for future efforts to investigate *T. gondii* in coastal waters in other locations and in other affected marine mammal species. Research in California has highlighted that sea otters are likely infected with *T. gondii* after consuming prey that contain oocysts that have been shed by a felid, transported from the land to the sea through runoff, and incorporated in the marine food web, so ultimately all steps in this chain must be evaluated and localised to specific watersheds and coastal segments when assessing risk of *T. gondii* infection (Burgess et al., 2013; Tinker et al., 2019). The southern sea otter monitoring program in California, instigated in 1992, is a

collaborative effort involving complete pathological examination of beachcast carcasses as well as capture, tagging and health screening of live, free-ranging sea otters (Conrad et al., 2005). As such, it is a model for investigating causes of mortality in threatened and endangered species. Unfortunately, it is one that is extremely difficult to replicate. Such close surveillance of a population is certainly not the norm, is logistically complex, and reliant on a large amount of funding.

In New Zealand, research groups exist which investigate disease and mortality in the endangered Hector's dolphin and New Zealand sea lion, which has led to the important discovery of toxoplasmosis as a cause of death for the aforementioned species. However, in contrast to the sea otter, live-sampling and tagging of a sufficient number of Hector's dolphins to draw statistically significant conclusions from seroprevalence studies is not a possibility. Furthermore, although stomach content and stable isotope analyses have been carried out to determine the Hector's dolphin diet (Miller et al., 2013; Miller, 2015), the fine-scale data permitted by tagging and live observation is infeasible in this species. Notably, Hector's dolphins predominantly eat fish and feed underwater. It would therefore be challenging to examine Hector's dolphin prey as *T. gondii* vectors and very difficult to determine whether dietary preferences are associated with differential risks of exposure to *T. gondii* within a locality for these dolphins, as seen in the sea otter.

Studies have shown that there are no standardised methods available to detect *T. gondii* oocysts in large volumes of water (see section 1.2.5.3 Shellfish as sentinels of *T. gondii* in the marine environment, pg. 54), meaning oocyst delivery to the ocean cannot yet be directly measured. However, in California, spatial patterns of sea otter seroprevalence are considered a proxy for the oocyst load in coastal waters (VanWormer et al., 2016). As this is not possible in New Zealand with respect to Hector's dolphins, alternative means to determine the prevalence of *T. gondii* in coastal habitats and risk factors for coastal contamination need to be employed, which could be addressed by using shellfish or other marine invertebrates as biosentinels, another method utilised in California (see section 1.2.5.3 Shellfish as sentinels

of *T. gondii* in the marine environment, pg. 54). To successfully evaluate all steps in the *T. gondii* transmission chain, investigation of oocyst shedding prevalence in New Zealand cats would also be pertinent in order to estimate the oocyst load in coastal waters around the country, methods discussed below in section 1.2.5.2 Cats and *T. gondii* transmission, pg. 44. Terrestrial-marine links may also be solidified by comparing *T. gondii* genotypes from Hector's dolphins with those from New Zealand cats and marine invertebrates.

1.2.5.2 Cats and *T. gondii* transmission

For risk assessment and control of *T. gondii* transmission, knowledge of feline infections and their effect on the oocyst burden of the environment is essential (Jokelainen et al., 2012). Knowledge of the distribution of *T. gondii* genotypes in cats is also essential to identify sources of infection for intermediate hosts as well as high-risk cat populations for management purposes (VanWormer et al., 2014). With the discovery of toxoplasmosis as a major cause of death for the Southern sea otter in California (Kreuder et al., 2003), there has been a particular research focus on felids that inhabit watersheds bordering the sea otter range as the likely source of infective oocysts (Dabritz et al., 2006, 2007a, b; Miller et al., 2008; Dabritz & Conrad 2010; VanWormer et al., 2013a, b, 2014, 2016; Burgess et al., 2018; Shapiro et al., 2019b), with biophysical studies suggesting that oocysts in contaminated runoff preferentially concentrate in nearby coastal habitats (Shapiro et al 2019a). Efforts have been made to gather baseline data on *T. gondii* prevalence in these cats, to consolidate the land-sea transmission hypothesis through molecular tracking, and to investigate feline-related factors that may impact sea otter exposure and mortality from toxoplasmosis. Recent sea otter related studies have identified feline shedding prevalence and cat densities as important predictors of sea otter exposure (VanWormer et al., 2016; Burgess et al., 2018). Moreover, in-depth molecular analyses, linking *T. gondii* genotype to mortality outcomes in sea otters, showed that virulent strains are also found in felids living nearby (Shapiro et al., 2019b).

1.2.5.2.1 Infection and shedding in felids

Toxoplasma gondii infection in cats can be determined by testing for anti-*T. gondii* IgG antibodies (see section 1.2.4.1 Serology, pg. 21) (Dubey et al., 1995; Barrs 2009). When applied at a group or population level, this can give a seroprevalence estimate. Serological surveys conducted worldwide have shown that *T. gondii* infection is endemic in wild and domestic cat populations (Dubey & Jones, 2008; Dabritz & Conrad, 2010; Lappin, 2010; Jokelainen et al., 2012). Global *T. gondii* seroprevalence is reported to be 30 – 40 % for domestic cats (Elmore et al., 2010; Dubey, 2016) but estimates vary widely between and within populations, regions, and countries. Seropositive cats are considered previous oocysts shedders (Jones & Dubey, 2010), thus seroprevalence estimates provide an important indication of the extent of environmental contamination (Dubey et al., 1995; Jokelainen et al., 2012). Indeed, in sea otter related research, Dabritz et al. (2007b) used seroprevalence data gained from domestic cats, combined with faecal deposition and population size estimates, to calculate the annual oocyst burden in the nearshore terrestrial environment adjacent to a high-risk sea otter site. Determining seroprevalence to be 29.6 %, the population of 7,284 owned cats was estimated to defecate about 1.1 billion oocysts into this 3,104-hectare coastal ecosystem annually. An environmental burden of 36 oocysts/m^2 over the region (likely more intense in areas with large cat populations) suggested that oocysts were present in sufficient numbers to reach marine waters, where they could be ingested by sea otters.

There are, however, some limitations associated with using seroprevalence data to estimate the environmental oocyst burden. Particularly, domestic and wild felids actively shedding oocysts can test seropositive or seronegative based on their route of exposure to *T. gondii*, which influences timing of shedding and antibody development (Dubey et al., 1970b; Dubey, 1976; Ruiz & Frenkel, 1980; Dubey et al., 1995a; Dubey, 1995; VanWormer et al., 2013a, 2013b). To circumnavigate this problem, it is possible to determine the incidence of infection (Afonso et al., 2006; Simon et al., 2018). However, this is impractical, requiring repeat sampling of serum from large populations of cats for a year or more (longitudinal studies) in order to demonstrate seroconversion (Dabritz & Conrad, 2010). Alternatively, estimates of terrestrial

contamination can more easily be gleaned through observational studies of the prevalence of oocyst shedding in cat faeces.

A number of studies have done exactly this, examining shedding prevalence by testing cat faeces for the presence of oocysts (reviewed by Dabritz & Conrad, 2010 and VanWormer et al., 2013b). This can be done using faecal floatation and microscopy, which may be coupled to bioassay for confirmatory purposes (e.g. McKenna, 1978; McKenna & Charleston, 1980). More recently, microscopy has been coupled with molecular techniques, particularly to distinguish between *T. gondii* and morphologically similar protozoan (oo)cysts that may be found in cat faeces, such as *Hammondia hammondi* (Schares et al., 2008a, b). *Toxoplasma gondii* oocyst shedding has now been identified in pet cats, feral domestic cats, and free-ranging individuals from diverse wild felid species (Dubey et al., 2009a; VanWormer et al., 2013a, b; Shapiro et al., 2019a, b). In naturally-infected pet and feral domestic cats, molecularly or bioassay confirmed shedding prevalence of *T. gondii* oocysts is reported to range from 0 – 20 % (as reviewed by VanWormer et al., 2013b). The prevalence of cats shedding oocysts in naturally-infected, free-ranging wild felid populations is reported to range between 0 – 37 % (Jokelainen et al., 2013; Simon et al., 2013b; VanWormer et al., 2013b).

Shedding prevalence estimates can be used to calculate the oocyst burden in a locality (Dabritz et al., 2007b; Dabritz & Conrad, 2010). For example, at a local level, Dabritz et al. (2007b) tested cat faeces for the presence of *T gondii* oocysts to help determine the proportion of cats shedding oocysts and calculate the number and density of oocysts entering the coastal environment. Only 3 of 326 (0.9 %) samples from cats in the Morro Bay area of California contained *T gondii*–like oocysts. Nevertheless, on the basis of the estimated quantity of cat feces deposited outdoors in this area, the annual burden in the environment was estimated to be 94 to 4,671 oocysts/m^2. The authors concluded that despite the low prevalence and short duration of *T. gondii* oocyst shedding by cats detected in their survey and former studies, the sheer

numbers of oocysts shed by cats during initial infection could lead to substantial environmental contamination. A national level estimate for the entire USA was later provided by Dabritz & Conrad (2010), along with an equation to estimate the density of oocyst loading (D) in the environment that could be applied to cat populations overseas:

$D = \{[(O \times \rho O) + E] \times \omega \times \rho T \times K\} / A$

where **O** = owned cat population size; **ρO** = proportion of owned cats defecating outside 100% of the time; **E** = feral and stray cat population size; **ω** = annual faecal production per cat of 14 600 g; **ρT** = proportion of cat faeces containing *T. gondii* oocysts; **K** = concentration of *T. gondii* oocysts in cat faeces (e.g. 1.56×10^5 oocysts/g for infections producing 50 million oocysts shed for 8 days) and **A** = land area. The output of such a calculation for the USA, assuming that feral cats comprised either 25 % or 45 % of the total cat population came to 779 – 1728 oocysts/m^2, again showing that cats that defecate outdoors may be capable of disseminating large quantities of oocysts into the environment.

As oocysts cannot replicate outside of the feline definitive host, factors that impact feline infections and shedding, along with felid population sizes, will impact the environmental oocyst burden. To prevent infection of cats and thereby reduce oocyst shedding, risk factors for infection need to be well understood. So far, factors known to affect seroprevalence include the age, sex, breed, and lifestyle of the cat, amongst others (Dubey, 1973; Vollaire et al., 2005; Afonso et al., 2006, 2010; Opsteegh et al., 2012; Must et al., 2015, 2017). A cat's lifestyle appears to be a particularly important predictor of infection, with engagement in hunting behaviour through outdoor access associated with higher odds of seropositivity (Opsteegh et al., 2012; VanWormer et al., 2013a; Must et al., 2015). As an example, Opsteegh et al. (2012) estimated *T. gondii* seroprevalence in owned domestic cats in the Netherlands to be 18.2 % (95 % CI: 16.6 – 20.0). Using multivariable logistic regression, the authors identified hunting, being a former stray cat, and being fed raw meat as risk factors; hunting contributed most to the *T. gondii* seroprevalence in the sampled population (35 %) and the odds of being seropositive were 4.1 times higher in cats that hunted compared with those

who did not. Similarly, VanWormer et al. (2013a) concluded that observed differences in infection prevalence between sympatric feral domestic and wild felids in coastal California were likely linked to diet. Unmanaged feral domestic cats, mountain lions (*Puma concolor*), and bobcats (*Lynx rufus*), which subsist primarily on wild prey, were found to exhibit high levels of *T. gondii* exposure (72.7 – 81.3 %). In contrast, a significantly lower seroprevalence (16.8 %) was detected in managed feral cats, considered likely to reflect their access to alternative food sources, such as commercial cat food or scraps scavenged from or distributed by people, which pose a lower risk of exposure to *T. gondii* tissue cysts.

Lifestyle and diet are also expected to strongly influence oocyst shedding (Afonso et al., 2007; Herrmann et al., 2010; VanWormer et al., 2013a; Shapiro et al., 2019a). In the study of VanWormer et al. (2013a), discussed above, oocyst shedding in overlapping populations of feral domestic and wild felids was investigated to estimate relative contributions to coastal oocyst loading. Unmanaged feral domestic cats and smaller wild felids (bobcats) feeding primarily on wild prey were over 13 times more likely to be shedding *T. gondii* oocysts than managed feral domestic cats being fed by humans ($p = 0.04$), attributed to their likely greater consumption of potential intermediate hosts. These dietary preferences may make them more likely to encounter infected intermediate hosts, which could lead to higher rates of initial infection and shedding. Observed shedding prevalence may also be higher in cats that consume a relatively large number of small prey items or prey species with higher prevalence of *T. gondii* infection because of increased exposure to new strains of *T. gondii* and repeat oocyst shedding. Oocyst shedding is often stated to occur only once in a cat's lifetime after initial infection, usually when a young cat is weaned and starts to hunt (Dubey & Frenkel, 1974; Fritz et al., 2012). Indeed, young cats may shed higher numbers of oocysts following primary infection and shedding prevalence has been observed to be higher in outdoor pet and feral kittens compared to adults (Ruiz & Frenkel, 1980; Dubey & Beattie, 1988). However, older cats may still play an important role in environmental loading, as oocyst shedding has been detected in naturally infected adult domestic cats (1 – 18 years old) in Europe and the United States (Schares et al., 2008b; Berger-Schoch et al., 2011; Jokelainen et al., 2012; VanWormer et al., 2013a). This may be due to repeat

shedding over a cat's lifetime, as evidenced by experimental and field studies (Dubey, 1976, 1995; Lukesova & Literák, 1998; Zulpo et al., 2018). Evidence suggests that re-shedding is more likely following co-infection with another common feline parasite, *Isospora felis*, after being treated with large doses of corticosteroids, as well as after re-infection with a different strain of *T. gondii* (Chessum, 1972; Dubey & Frenkel, 1974; Dubey, 1976; Zulpo et al., 2018). Interestingly, the strain of *T. gondii* infecting a cat may impact both the number of oocysts shed by an individual cat as well as the prevalence of oocyst shedding (Shapiro et al 2019a). Overall, cats of all of ages should be considered a potential source of oocysts due to the possibility of re-shedding (Shapiro et al 2019a), which may in turn be influenced by hunting behaviour and diet.

1.2.5.2.2 Genotypes of *T. gondii* in felids

Efforts have also been made to characterise genotypes of *T. gondii* associated with feline infections, in order to molecularly track the parasite from land to sea (Miller et al., 2008; VanWormer et al., 2014). Particularly, genotypes associated with feline infections have been compared to those infecting the sea otter (Miller et al., 2008; VanWormer et al., 2014), and those contaminating naturally exposed California mussels sampled from sea otter habitat (Miller et al., 2008; Shapiro et al., 2015).

The vast majority of sea otter infections are caused by atypical Type X-related (ToxoDB #4, #5) and Type II-related strains (ToxoDB #1, #3) (Miller et al., 2004, 2008; Sundar et al., 2008; Shapiro et al 2019b), and similar strains also seem to be highly prevalent in cats living near sea otter habitat (Miller et al., 2008; VanWormer et al., 2014). The study of VanWormer et al. (2014) provides the latest and most comprehensive survey of coastal felids in California. In this study a large number of animals from sympatric populations of domestic cats and wild felids were targeted, including unmanaged feral domestic cats (*n* = 166), bobcats (*n* = 27), and mountain lions (*n* = 73), with tissue samples collected at necropsy. Samples were tested for *T. gondii* by PCR and genotypes were characterised using PCR-RFLP and direct DNA sequencing at six polymorphic loci, namely B1, SAG1, SAG3, GRA6, L358, and Apico. *Toxoplasma gondii*

DNA was detected in samples from 70 (26 %) individual cats, and genotyping revealed archetypal and atypical alleles, predominantly belonging to Type II *T. gondii* or Type X. Moreover, a spatial association between genotypes in coastal felids and sea otters was identified – spatial clusters of archetypal (Type II) and atypical (Type X) infections in felids were observed along the coast, bordering previously identified clusters of Type II and Type X *T. gondii* infections in sea otters. Evidence therefore suggests that marine mammals that live close to the shore are exposed to the same strains of *T. gondii* as found in nearby cats, supporting a local land-sea connection.

To confirm whether virulent strains of *T. gondii* could be traced back to coastal felids, using fine-scale genotyping Shapiro et al. (2019b) compared sea otter isolates with those previously reported by VanWormer et al. (2014). Importantly, a Type X variant strain isolated from five out of 12 (42%) sea otters diagnosed with toxoplasmosis as the primary cause of death was identified in two feral domestic cats and a bobcat. An identical Type X variant SNP was identified in all the isolates at the B1 gene, strongly indicating that virulent genotypes are linked from source (felids) to host (sea otters) from land to sea in California. This link is further strengthened by the discovery of the same genotype in naturally exposed shellfish collected from otter habitat (Shapiro et al., 2015), discussed in more detail in section 1.2.5.3 Shellfish as sentinels of *T. gondii* in the marine environment, pg. 54.

Another objective of feline studies in California was to identify high risk cat populations or habitats by investigating the distribution of genotypes in felids living adjacent to otter habitat (Miller et al., 2008; VanWormer et al., 2014), particularly to work out whether certain cat populations were the source of Type X-related strains. In VanWormer et al. (2014), the authors used multivariate logistic regression to investigate risk factors for Type X infection in coastal felids, including cat type (domestic or wild), age class (juvenile or adult), sex, season (wet or dry), year of sampling, and predominant land use (developed vs. undeveloped) within 5 km of the sampling location. Results showed that there was a significant difference

in the proportion of archetypal and atypical genotypes between domestic cats and wild felids. Wild felids were five to 14 times more likely to be infected with *T. gondii* strains consistent with Type X than feral domestic cats, which were mainly infected with strains consistent with Type II. Furthermore, adjusting for cat type, Type X infections were more likely to occur in cats living in undeveloped lands, whilst Type II infections were more likely in developed areas. Interestingly, results appeared to show that in coastal California there exists separate domestic and wild (sylvatic) cycles of *T. gondii* transmission, whereby archetypal genotypes predominate in domestic species and humans, whilst a more diverse range of genotypes, including atypical strains, predominate in wildlife.

Despite this, there seems to be overlap in sylvatic and domestic transmission cycles in California, as strains consistent with Type X were detected in 22 % (11/49) of the *T. gondii*-positive feral domestic cats, some of which were later matched to strains from fatal toxoplasmosis sea otter cases (Shapiro et al., 2019b, see above). As such, the domestic cat cannot be discounted as a source of this virulent genotype for the sea otter. It should be noted, however, that the studies of Miller et al. (2008) and VanWormer et al. (2014) characterised genotypes of *T. gondii* from feline tissue samples, not oocysts. Furthermore, owned (pet) domestic cats and strays were not included in these studies, meaning genotypes infecting and shed by this large groups of cats remain to be determined. Considering that the owned and stray cat population in coastal California is estimated to be 75 times larger than wild felid population (VanWormer et al., 2013a), and is closely linked to the human population, it is possible that these cats are an important source of sea-otter virulent genotypes in developed areas. Indeed, the estimated numbers of infected and shedding outdoor pet cats and managed feral cats were found to be drastically higher than those of unmanaged feral cats, mountain lions, and bobcats (VanWormer et al., 2013a). Moreover, cat density has been found to be an important predictor of sea otter exposure to *T. gondii* (VanWormer et al., 2016; Burgess et al., 2018): higher levels of *T. gondii* infection were documented in sea otters living to areas with greater human presence where oocyst contributions were estimated to be highest for outdoor pet cats and managed feral cats (VanWormer et al., 2016; Burgess et al., 2018). Hence, pet and stray cats may be less infected with

Type X but appear to contribute considerably more oocysts than unmanaged feral cats and wild felids. As such, even in localities where wild felids are found, such as coastal California, domestic cats, whether owned, stray, or feral, play an important role in land-sea transmission of *T. gondii*. With a growing human population and coastal development, this problem is likely to be exacerbated with a rising number of domestic cats inhabiting the land and possible merging of sylvatic and domestic cycles promoting the spread of 'wild' genotypes (VanWormer et al., 2016; Shapiro et al 2019a, b).

It is not known if strains causing toxoplasmosis in marine mammals in places where there are no wild felids, such as New Zealand, are more likely to come from owned, stray, or feral domestic cats, or whether from cats inhabiting developed or undeveloped landscapes. Indeed, at this point in time, although there are many studies investigating *T. gondii* in felids across the globe (summarised in Amouei et al., 2020), none appear to examine the distribution of genotypes in feline infections, taking into account cat type and habitat. It is also not clear whether independent sylvatic cycles persist in areas where few to no wild felids live. On the one hand, in Europe where there are only three species of wild felid, the domestic Type II lineage is found to dominate in both wild and domestic animals (Galal et al 2019), including pet and stray cats (Schares et al., 2008; Herrmann et al., 2010; Jokelainen et al., 2012). On the other, in Australia where there are no wild felids, authors report a possible sylvatic cycle in wildlife which may promote the transmission of Type II-like strains with unique polymorphisms at the B1 gene (Parameswaran et al., 2010; Donahoe et al., 2014). Such strains have been identified in marine mammals in the country, resulting in fatal toxoplasmosis in a New Zealand fur seal (Donahoe et al., 2014) and a Risso's dolphin (Cooper et al., 2016). With respect to domestic animals, a small study of Australian pet cats (n = 8) found that half of the animals were infected with Type II (ToxoDB #3) *T. gondii*, with no unique polymorphisms observed at any of the typing loci, which included B1, C22-8, GRA6, C29-2, L358, PK1, alt.SAG2, SAG2, SAG3, BTUB, and Apico (Brennan et al., 2016). Interestingly, the other half of the animals mainly carried Type II alleles but with unique polymorphisms identified, primarily at the B1 locus (three out of four cats). Furthermore, a Type-II like strain with a specific B1 polymorphism (U-6) isolated from one pet cat was previously identified in a

goat from Tasmania as well as in a wombat which had severe neurological symptoms before death (Parameswaran et al., 2010). As such, it is not yet clear whether distinct sylvatic and domestic cycles exist in Australia, and although owned cats clearly can be a source of atypical strains, it is not known whether these strains are more prevalent in stray or feral cats, or whether atypical Type II-like strains are just widespread in the country. It is also worth noting that studies that include additional typing loci, notably the B1 gene, and make use of sequencing as well as (virtual) RFLP, tend to find a higher prevalence of atypical genotypes. For example, the New Zealand fur seal (Donahoe et al., 2014) and Risso's dolphin in Australia (Cooper et al., 2016) would be classified as variant Type II (ToxoDB #3) if the B1 locus had not been sequenced.

1.2.5.2.3 Cats in New Zealand

New Zealand provides an unusual research context as there are no wild felids in the country and the only native mammals are marine mammals and bats; a wide range of unique avifauna occupy the ecologic niches normally filled by mammals (King, 1990; Howe et al., 2014). Mammals, including the domestic cat, mice and rats, were introduced to New Zealand with the arrival of Maori in the 14th century and European settlers in the mid-19th century (King, 1990; Howe et al., 2014). Since then, the size of the cat population in New Zealand has significantly increased and pet cat ownership is now estimated at 1.1 million animals (New Zealand Companion Animal Council, 2016; Zito et al., 2019). A very high proportion of pet cats are allowed outdoors in New Zealand compared with other countries in the world (Farnworth et al., 2010; Hall et al., 2016), where they can hunt and deposit faeces, which may increase the risk of environmental oocyst contamination. There is also thought to be a substantial number of stray and unmanaged feral cats, although density estimates vary (Farnworth et al., 2013). Despite growing evidence of toxoplasmosis as problem for native wildlife (Roe et al., 2013, 2017; Howe et al., 2014; Michael et al., 2016), particularly the Hector's dolphin, as well as important livestock species (Patel et al., 2017), very little is known about *T. gondii* in cats in New Zealand and there is limited baseline data available on the number of cats shedding *T. gondii* oocysts and the genotypes of *T. gondii* circulating in these animals. Particularly, it is not yet known

whether the variant Type II *T. gondii* genotypes that have been linked to toxoplasmosis deaths in Hector's dolphins and other native wildlife, can be traced back to specific cat populations or habitats.

1.2.5.3 Shellfish as sentinels of *T. gondii* in the marine environment

Given that there is considerable dilution of runoff and pathogens on delivery to coastal waters, widespread infections in diverse marine mammal species has puzzled researchers (Shapiro et al., 2015). With the discovery of toxoplasmic encephalitis as a major cause of death for the southern sea otter in California (Cole et al., 2000; Kreuder et al., 2003), efforts were made to identify the source of infection and clarify transmission routes, as discussed above. Noting that filter-feeding marine bivalves are a major prey species of Southern sea otters (Kvitek et al., 1988), experimental studies by Lindsay et al. (2001, 2004) and Arkush et al. (2003) were pivotal, confirming that sporulated *T. gondii* oocysts can be concentrated by marine oysters and mussels and remain infectious for laboratory mice. These findings suggested that naturally exposed shellfish could indeed be paratenic hosts of *T. gondii* in coastal ecosystems. Long-term infectivity of sporulated oocysts in shellfish was established by Lindsay et al. (2004). The authors exposed eastern oysters (*Crassostrea virginica*) to 1 x 10^6 oocysts for 24 h, after which whole oysters were homogenised, 1 to 85 days post-exposure, and fed to CD-1 mice. Two out of three oysters tested were found to be bioassay positive 85 days after exposure.

The experimental study of Arkush et al. (2003) also laid the groundwork for future studies by establishing that PCR is a suitable method for detecting *T. gondii* in shellfish. Notably, the authors found that *T. gondii* could be detected in all mussel tissues as soon as 3 h post-exposure, and for up to 7 days in the digestive gland, using their TaqMan qPCR targeting *T. gondii*-specific ssrRNA. In a separate experiment lasting 35 days, where mussels were exposed to 1.5 x 10^7 oocysts for 6 h, the authors found that *T. gondii* ssrRNA could be detected up to 21 days after exposure, in both haemolymph and digestive gland, suggesting that as the concentration of oocysts increases, the longer mussels remain positive for *T. gondii* post-exposure.

Whilst Arkush et al. (2003) and Lindsay et al. (2004) assessed *T. gondii* detection during shellfish depuration after a one-off exposure to oocysts, Palos Ladeiro et al. (2014, 2015) assessed detection during continuous exposure, as well as detection during depuration, using freshwater zebra mussels (*Dreissena polymorpha*). In their earlier work, these authors used a TaqMan qPCR targeting the rep529 marker of *T. gondii* to show that laboratory-exposed zebra mussels could accumulate *T. gondii* oocysts simultaneously with (oo)cysts of *Cryptosporidium* spp. and *Giardia* spp., closely related protozoans that are also known to contaminate aquatic environments. The authors reported a dose-response relationship; 50 % of mussels exposed to 20 *T. gondii* oocysts per mussel per day over a seven-day period were found to be positive for *T. gondii* DNA but 100 % of mussels exposed to 200 or 560 oocysts per mussel per day over the same time period were PCR positive. Using qPCR, they found that the number of oocysts increased in line with increasing ambient concentration. Overall, these studies provide experimental evidence to suggest that shellfish can concentrate and transfer *T. gondii* oocysts to higher trophic levels in the marine and freshwater environments, implying that they could be a source of *T. gondii* infection for the southern sea otter and other consumers. Furthermore, these experimental studies suggest that testing shellfish for the presence of *T. gondii* using PCR is an effective means to carry out surveillance for *T. gondii* oocyst pollution in coastal waters.

Indeed, at present, a surveillance approach using shellfish as biosentinels of *T. gondii* contamination in marine habitats appears optimal over testing seawater samples directly (Shapiro et al., 2015). Although Lindsay & Dubey (2009) showed experimentally that *T. gondii* oocysts can sporulate and survive long-term in seawater and numerous methods for *T. gondii* detection in water have been described (e.g. Shapiro et al., 2010, reviewed in Bahia-Oliveira et al., 2017), few investigations have attempted to test seawater for the presence of the parasite. In fact, *T. gondii* presence in environmental samples collected from marine ecosystems has not yet been confirmed (Kourenti & Karanis, 2006; Shapiro et al., 2010; Verant et al., 2014). In general, methods used to detect *T. gondii* oocysts in water require the filtration of large volumes of water due to the relatively dilute distribution of *T. gondii* oocysts in environmental water sources, which

can lead to a loss of target organisms (Shapiro et al., 2010, 2019a; Staggs et al 2015; Kerambrun et al., 2016). In addition, water-testing methods that rely on microscopy-based detection require molecular confirmation to definitively identify oocysts as *T. gondii*, because oocysts of other apicomplexan parasites have a similar morphology (e.g., *Hammondia* spp., *Besnoitia* spp., and *Neospora* spp.) (Shapiro et al., 2019a). Although *T. gondii* oocyst-like structures have been observed in estuarine waters off the Galapagos Islands, they could not be molecularly confirmed (Verant et al., 2014). Therefore, surveillance at this point in time using biosentinels seems to be expedient to investigate the extent of, and risk factors associated with, nearshore contamination with *T. gondii* oocysts.

Special attention has been paid to filter-feeding bivalve shellfish, such as mussels, oysters, and clams, as biosentinels because of their ability to continuously filter large volumes of water and accumulate suspended particles, including pathogens, from water (Graczyk et al., 1999; Miller et al., 2005; Palos Ladeiro et al., 2014; Shapiro et al., 2015). With respect to *T. gondii*, shellfish may offer a particularly efficient approach for marine ecosystem monitoring; uptake of *T. gondii* oocysts by coastal invertebrates (and fish) may be facilitated by certain surface properties of oocysts, a phenomenon investigated by Shapiro et al. (2009, 2012b, 2015). Shapiro et al. (2009) evaluated the surface properties (electrostatic charge and affinity for water) of *T. gondii* oocysts in an effort to understand the transport behavior of oocysts from overland freshwater runoff to coastal waters. In particular, the authors found that oocysts are negatively charged and hydrophilic whilst in freshwater, meaning they are easily drawn into and transported in waterways and can bypass commonly used treatment processes. In estuarine and saltwater, however, oocysts lose their negative charge and as oocysts move into saline water they more readily clump together (flocculate) and associate with macroaggregates, or 'marine snow' (Shapiro et al., 2012b, 2015). This increased association of oocysts with marine snow increases their bioavailability to bivalves, other invertebrates, and fish (Shapiro et al., 2015) as particles are more readily ingested within aggregates than particles freely suspended in the water column. Most bivalves feed on phytoplankton aggregates or sedimented organic material, preferring near-shore or shallow waters where nutrient levels are high and

where the concentration of *T. gondii* oocyst pollution in runoff is likely to be highest (Robertson, 2007). Moreover, shellfish are generally sessile and are relatively easy to collect in large numbers (Palos Ladeiro et al., 2014; Staggs et al., 2015), making them excellent biosentinel candidates.

Recently, as many marine mammals are piscivorous and do not usually consume shellfish, fish have also been investigated as potential paratenic hosts of *T. gondii*. Through laboratory studies, Massie et al. (2010) demonstrated that migratory filter feeding fish, specifically northern anchovies (*Engraulis mordax*) and Pacific sardines (*Sardinops sagax*), could filter *T. gondii* oocysts from water. Using PCR, they showed that oocysts persisted in the fish for at least 8 h post-exposure, and mouse bioassay experiments with sardines demonstrated that the oocysts remained infectious inside the fish's alimentary canals. Since then, *T. gondii* DNA has been detected in naturally exposed fish in China (Zhang et al., 2014) and Italy (Marino et al., 2019). Utilising qPCR as well as recently developed digital PCR (dPCR), Marino et al. (2019) discovered that 32 out of 147 pooled samples from 12 different fish species were contaminated with *T. gondii* DNA, detected in 16 samples of skin/muscle and in 11 samples of both intestine and gills. These studies represent an extremely important approach to understanding transmission of *T. gondii* to marine mammals, particularly as some fish species migrate between fresh- and saltwater environments, or between nearshore and pelagic zones, suggesting they could be an efficient vector for piscivorous marine mammals like cetaceans (Simon et al., 2013a). However, these characteristics do mean that fish may not be optimal as biosentinels, as their mobility means that it may be harder to ascertain where they were contaminated, whilst bivalves are sedentary and can even be outplanted for surveillance purposes (Miller et al., 2002a, 2008).

Shellfish have been used for many years as bioindicators of aquatic contamination with heavy metals and pesticides (O'Connor, 2002). For example, since 1986, NOAA's Mussel Watch Program has monitored coastal waters across the United States for chemical contaminants and biological indicators of water quality using bivalves as sentinels (Kimbrough et al., 2008; Staggs et al., 2015). Shellfish have also been recognised

worldwide as sentinels of aquatic contamination with faecal bacteria, viruses, and parasites, including protozoan parasites of terrestrial origin, namely *Cryptosporidium* spp. and *Giardia* spp. as well as *T. gondii* (Fayer et al., 1998; Miller et al., 2005; Putignani et al., 2011; Willis et al., 2013; Ghozzi et al., 2017). Although research on shellfish and other marine invertebrates contaminated with *T. gondii* represents a relatively new field of study (Shapiro et al., 2019a), to date, testing of filter-feeding bivalves as sentinels for pollution has found marine ecosystem contamination with *T. gondii* in various countries, including the USA, Turkey, Tunisia, Brazil, and China (Esmerini et al., 2010; Miller et al., 2008; Putignani et al., 2011; Aksoy et al., 2014; Zhang et al., 2014; Shapiro et al., 2015; Staggs et al., 2015; Ghozzi et al., 2017) (recently reviewed by Bahia-Oliveira et al., 2017, Shapiro et al., 2019a). Contaminated wild and commercially purchased shellfish have been reported. Prevalence of shellfish contamination ranges from a low of 0.07 % (1/1396) (Miller et al., 2008) to a high of 54 % (14/26) (Staggs et al., 2015) (summarised in Bahia-Oliveira et al., 2017).

Considering the use of shellfish to investigate land-sea transmission of *T. gondii* to marine mammals specifically, a field study conducted by Miller et al. (2008) was integral to understanding parasite transmission to the Southern sea otter in California. This study confirmed, for the first time globally, the presence of *T. gondii* in naturally exposed shellfish, and in areas that were previously reported as high-risk for sea otter infection with *T. gondii* (Miller et al., 2002a, 2004). In more detail, the study was the first to undertake a comprehensive survey of wild invertebrates in the marine environment. Between 2002 and 2004, 1396 marine and estuarine invertebrates, including mussels (*Mytilus californianus*), were sampled from nearshore marine waters along the central California coast. Of these, one mussel was confirmed to be harboring *T. gondii*, with *T. gondii* ssRNA detected in digestive gland by TaqMan 18S rRNA qPCR and direct sequencing. One resident sand crab (*Emerita analoga*) was also found to positive by qPCR but this result could not be confirmed by sequencing or genotyping due to a lack of remaining sample. Furthermore, previous research had found that the majority (over 72 %) of *T. gondii*-positive otters in the area were infected with Type X (ToxoDB #5) (Conrad et al., 2005). In Miller et al. (2008), authors were able to genotype the strain of *T. gondii* in the positive mussel, accomplished via multilocus PCR–RFLP analysis at the

B1 and SAG1 loci, followed by DNA sequencing of B1 and SAG1 amplicons for comparison with published *T. gondii* sequences. They found that the mussel was contaminated with the same Type X genotype as seen in the majority of otter infections, indicating that consumption of wild invertebrates is a plausible route of infection for these animals, particularly as sea otters tend to feed directly along the shoreline, often within or adjacent to stormwater discharges, and consume 25 % of their bodyweight in prey each day.

Since then, large-scale live sampling and behavioural studies have shown that consumption of marine snails, rather than mussels, is significantly associated with a high risk of *T. gondii* infection in the southern sea otter (Johnson et al., 2009; Krusor et al., 2015; Burgess et al., 2013; Tinker et al., 2019), although *T. gondii* has not yet been detected in local naturally exposed marine snails (Krusor et al., 2015). Despite this, mussels remained an important focus of research in California, later used as sentinels for *T. gondii* in sea otter habitats to further elucidate risk factors for marine contamination (Shapiro et al., 2015; Staggs et al., 2015). Examining these studies in more detail is useful; while the health implications of coastal habitat contamination with *T. gondii* to marine wildlife and humans have been described, reports documenting the extent of, and risk factors associated with, nearshore contamination with the parasite remain relatively scarce. In particular, Shapiro et al. (2015) used validated, optimised molecular methods to evaluate the distribution and molecular epidemiology of *T. gondii* contamination along the central California coast, by means of surveillance in mussels. Specifically, nested PCRs targeting the ITS1 and B1 genes were applied for *T. gondii* detection in haemolymph samples from 959 wild mussels (*M. californianus*). A total of 13 mussels (1.4 %) tested positive for *T. gondii* DNA, confirmed by direct sequencing. Mussels were sampled from two regions documented as high risk for *T. gondii* exposure and/or death in California sea otters (Miller et al., 2002a; Kreuder et al., 2003; Shapiro et al., 2012a) along a similar stretch of the coast as in Miller et al. (2008). Results showed that the odds of detecting *T. gondii* were more than 12 times higher in mussels sampled during the wet season (November to May) as compared with the dry season (June to October) (univariable logistic regression odds ratio (OR) 12.3, 95 % confidence interval (CI) 1.6 – 94.6, $P = 0.016$), and nearly five times higher in mussels collected near freshwater outflow (OR 4.9, 95 % CI 1.1 – 22.2, $P = 0.040$).

Previously, Miller et al. (2008) had collected mussels during the wet and dry seasons, and other invertebrates during the wet season alone. Interestingly, both the positive mussel and sand crab in that study were collected just after the first significant rainfall event occurred, early in the wet season (December), which would have carried faeces to the ocean.

In Shapiro et al. (2015), molecular characterisation was possible for eight of thirteen mussels positive for *T. gondii* DNA, using PCR-RFLP and DNA sequencing on B1 or SAG1 PCR products. As in Miller et al. (2008), Type X (ToxoDB #5) *T. gondii*, previously detected in California carnivores and sea otters (Miller et al., 2004, 2008; VanWormer et al., 2014), was observed at the B1 locus in three mussels. Three other distinct RFLP banding patterns were observed at the same locus, including those consistent with *T. gondii* Type I (two mussels), Type II/III (one mussel). An atypical RFLP digestion pattern was detected in two mussels which was identical to those obtained from five carnivores (two domestic cats, one mountain lion, and two foxes) sampled from nearby habitats in central coastal California (VanWormer et al., 2014). In addition to PCR-RFLP, DNA sequence analysis of the atypical alleles revealed further genetic variability at SNP sites that were not cleaved by restriction enzymes using the RFLP approach. Identical or extremely similar B1 sequences were present in the two wild mussels as in two wild carnivores (one mountain lion and one fox) sampled along the central Californian coast. Although full characterisation of genotypes across all typing loci was not achieved, finding an identical and atypical *T. gondii* allele in mussels and in felids from land that borders the same coastal waters strongly indicates that land-to-sea transmission of novel *T. gondii* strains is occurring through overland run-off, as highlighted by the authors. This work also provided novel evidence for the simultaneous presence of multiple *T. gondii* strains in mussels at a single coastal site, suggesting that faeces from multiple felids makes its way into ocean waters.

Published around the same time as Shapiro et al. (2015), Staggs et al. (2015) optimised and validated alternative PCR assays and conducted a small survey of select areas of the central California coastline for

the prevalence of *T. gondii* (and *Cryptosporidium* oocysts). In this study, *M. californianus* mussels were sampled from two sites and tested for *T. gondii* using a conventional PCR targeting the rep529 marker (Homan et al., 2000). An unexpectedly high prevalence was found, with a total of 19/41 (54 %) mussels testing positive for *T. gondii* DNA. Results led authors to question why much higher levels of detection were observed than reported by Miller et al. (2008) (0.09 %). In fact, although sampling from same stretch of coast, both Shapiro et al. (2015) and Staggs et al. (2015), found significantly higher prevalences of *T. gondii* in California mussels compared to Miller et al. (2008). Considering why this may be the case provides important insights for future studies in California and overseas. It is possible that the considerably higher prevalence in Staggs et al. (2015) could be due to the small sample size tested and specific targeting of wet season mussels - in that study mussels were only collected in April (year unknown), considered to be the rainy season in California, rather than year-round. However, importantly, the increased levels of detection in both of the later studies could be attributed to greater method sensitivity, i.e. ability to detect lower levels of *T. gondii* contamination, highlighting the need to carefully consider the analytical sensitivity and specificity (ASe, ASp) of an assay before implementation.

1.2.5.3.1 Challenges facing *T. gondii* detection in shellfish

These studies, in conjunction with other research, such as Esmerini et al. (2010), Hohweyer et al. (2013), Palos Ladeiro et al. (2015), and Manore et al. (2019), highlight problems likely to be faced when testing shellfish, show the importance of validating tests before conducting field surveys, and how this may be carried out. As Shapiro et al. (2015, 2019a) stress, methods for detecting *T. gondii* in shellfish are inconsistent across studies, which hinders direct comparison of prevalence and distribution. Studies utilise a variety of testing matrices, including whole tissue homogenates, gills, digestive tissues, or hemolymph. Importantly, different shellfish tissues appear to be more or less inhibitory for downstream molecular testing, which can confound study results (Staggs et al. 2015). It is thought that polysaccharides are primarily responsible for PCR inhibition in seafood but in bivalve shellfish, tissue glycogen content can also influence PCR efficiency (Schrader et al., 2012). Staggs et al. (2015) compared PCR inhibition between gill,

digestive gland, and haemolymph, using oocyst spiking experiments, and found detection was inhibited in digestive gland and gill tissue but not in haemolymph. Detection in more inhibitory matrices was improved by employing commercial inhibitor removal technologies, or by diluting DNA extracted from these tissues, but these options are not ideal for field studies as there may be a trade-off between detection and test sensitivity, and parasite numbers are likely to be low in naturally-exposed shellfish (Hohweyer et al., 2013). An immuno-magnetic separation technique has been developed to concentrate *T. gondii* oocysts (Hohweyer et al., 2016) but at present it lacks sensitivity and has not been validated for use in shellfish. Inhibition can be assessed using internal or external amplification controls (e.g. Miller et al., 2008; Tedde et al., 2019). However, this increases cost and with internal controls, competitive inhibition can occur, which may be a particular problem when target DNA levels are relatively low (Hoorfar et al., 2004). Overall, it seems the use of haemolymph is the current best option for shellfish testing as it does not appear to be inhibitory for downstream molecular work, recognising that prevalence may still be underestimated as oocysts may concentrate in different tissues depending on time since exposure (Arkush et al., 2003; Lindsay et al., 2004; Palos Ladeiro et al., 2014, 2015).

Using PCR assays targeting different markers of *T. gondii* can also affect test characteristics (as discussed in the diagnostics section). Considering prior research, the optimal way to validate and compare assays for *T. gondii* detection and genotyping in shellfish appears to be through oocyst spiking experiments, where known quantities of oocysts are spiked into the shellfish tissue of choice and analysed to determine the lowest concentration of oocysts that can be consistently detected amongst replicates (Esmerini et al., 2010; Shapiro et al., 2015; Staggs et al., 2015). Although the traditional approach for validating *T. gondii* PCR assays uses genomic DNA or plasmids to determine ASe (e.g. Kasper et al., 2009), oocyst spiking intuitively makes more sense for shellfish research as oocysts are the life-stage of *T. gondii* known to contaminate the marine environment. As highlighted by Shapiro et al. (2015), few comparative shellfish studies have yet been published in the literature, suggesting further work is needed in this area. The most sensitive assay validated to date appears to be a TaqMan qPCR targeting the rep529 marker of *T. gondii*, assessed by

Staggs et al. (2015), using known quantities of oocysts spiked into mussel haemolymph. It is important to note that DNA extraction procedures differ between studies, which may also affect test characteristics, particularly as the *T. gondii* oocyst has a tough exterior wall that must be penetrated before nuclear material can be accessed (Staggs et al., 2015; Manore et al., 2019).

Another issue faced by shellfish studies is that genotyping using the typing loci of Su et al. (2010) and PCR-RFLP, whilst possible, can be challenging. For example, whilst Staggs et al. (2015) reported a high prevalence of *T. gondii* DNA detected in study mussels, the authors could not successfully genotype any of the positive isolates using single copy RFLP markers. Oocyst spiking experiments revealed that RFLP typing could only be accomplished if the amount of gDNA present in a sample was equivalent to at least 1000 oocysts. As such, the lack of success in *T. gondii* genotyping of positive mussel samples was attributed to a combination of low oocyst numbers and low copy numbers of the target loci used for genotyping. Similar conclusions were drawn by Shapiro et al. (2015) who showed, when testing samples in triplicate, the lowest limits of *T. gondii* detection per millilitre haemolymph were 5 oocysts for detection targets ITS1, B1 and RE, but 50 oocysts for the SAG genes; and 100 oocysts for GRA6. So far, *T. gondii* genotypes have only successfully been characterised in aquatic invertebrates by targeting the multi-copy B1 gene, which is usually used for detection purposes, and subjecting amplicons to RFLP and/or direct sequencing. Genotypes reported include archetypal Type I (California, Turkey and Italy) (Aksoy et al., 2014; Marangi et al., 2015; Shapiro et al., 2015), Type II (California) (Shapiro et al., 2015), as well as atypical genotypes including Type X, as discussed above (Miller et al., 2004, 2008; Shapiro et al., 2015).

1.2.5.3.2 Determining infectivity and viability of *T. gondii* oocysts in shellfish

Although *T. gondii* nucleic acids have now been detected in field-sampled shellfish from around the world, it has not yet been possible to prove that they were contaminated by viable or infectious oocysts. This is necessary to confirm the marine transmission pathway and to definitively establish wild shellfish as a public

health threat for consumers, particularly considering that the infectious dose for *T. gondii* oocysts may be low (Dubey, 1996, 1997; Dubey et al., 1996). Hence, the risk of infection following ingestion of water or prey contaminated by low numbers of oocysts could be significant. Good working definitions of infectious versus viable *T. gondii* oocysts are provided by Rousseau et al. (2018), following Robertson & Gjerde (2008). An infectious oocyst of *T. gondii* is defined as a viable oocyst that is able to excyst and release its sporozoites, which can then infect host cells and differentiate within them into tachyzoites. In comparison, a viable oocyst is defined as being alive but not necessarily infective if its sporozoites fail to replicate in the host or to cause infection in susceptible cells. Viable parasites are potentially infectious, so viability is also a major feature for assessing health risks.

As discussed earlier, there are many different molecular targets specific to *T. gondii,* but these targets do not differentiate between genetic material of tachyzoites, bradyzoites, and oocysts. In addition, existing PCR assays used in shellfish do not determine whether *T. gondii* oocysts are sporulated, thus potentially infectious, and, since DNA can persist for a long time in dead cells, cannot distinguish between viable and dead parasites. Rousseau and colleagues (2018) reviewed the advantages and limitations of infectivity and viability assays to evaluate the risk of protozoan (oo)cysts in food, including *Cryptosporidium* spp., *Giardia* spp., and *T. gondii*. The following paragraphs briefly describe methods currently available to evaluate infectivity and viability of *T. gondii* oocysts, focusing on those that may be of particular use for shellfish testing.

At present, the gold standard test for determining oocyst infectivity is considered to be the bioassay (Rousseau et al., 2019a). Bioassays involve the inoculation (oral or parenteral) of susceptible animals with a sample thought to contain infectious oocysts, subsequent testing of the inoculated host for seroconversion, histopathological demonstration of *T. gondii* infection postmortem, and/or oocyst shedding in cats (Rousseau et al., 2019a). However, to date, only one report has described successful isolation of *T. gondii*

from water implicated in an outbreak of waterborne toxoplasmosis using bioassays in chickens and pigs (De Moura et al., 2006). Furthermore, although mouse bioassays have established the ability of different shellfish species to accumulate infectious oocysts under experimental settings (Arkush et al., 2003; Lindsay et al., 2004), these assays have not been successfully utilised in field studies. Only the study of Esmerini et al. (2010) reports an attempt to use bioassay to evaluate the infectivity of *T. gondii* in field-sampled oysters and mussels. The authors tested tissue homogenates from 60 groups of 5 oysters and 20 groups of 15 mussels using a nPCR targeting the B1 gene of *T. gondii* (Yai et al., 2003) and found two groups of oysters to be positive for *T. gondii* DNA (3 % of the oyster groups). However, all oyster and mussel groups were negative by mouse bioassay. The authors also used oocyst spiking experiments to determine the ASe of their mouse bioassay as well as their nPCR methods. For mussels, the ASe of the mouse bioassay was $\geq 10^3$ oocysts spiked into tissue homogenate (2/8 mice were positive for *T. gondii* infection after inoculation), whilst for oysters the ASe was $\geq 10^2$ oocysts. In contrast, the ASe of the nPCR for mussels was $\geq 10^2$ oocysts for mussels (3/10 replicates) but $\geq 10^3$ for oysters (6/15 replicates). The authors concluded that the negative bioassay results could be due to the presence of unsporulated or non-viable oocysts in the PCR-positive oysters, an insufficient inoculum volume, or the relative insensitivity of the bioassay compared to nPCR for oysters. At this point, it is not known whether the seeming lack of use of bioassays in field studies reflects a lack of reporting of unsuccessful attempts to use bioassays with shellfish, whether bioassay methods are widely unavailable, or if authors perceive limitations of bioassay methods to be too great. General limitations of bioassay methods include the involvement of animal ethics and expense. Bioassays are also time-consuming and labour/skill-intensive, taking weeks for results to become available. As such, they are not suitable in outbreak situations, or for assessing large numbers of samples. Results may be affected by inoculation route and *T. gondii* strain type (Arkush et al., 2003) and are qualitative (Hohweyer et al., 2013). As seen in Esmerini et al. (2010), another limitation of mouse bioassay is that the method may lack reliability when parasite numbers are low. As an example, in naturally exposed pigs, the density of *T. gondii* may be as low as one tissue cyst in 100 g of tissue. In one study, hearts of 1000 naturally exposed pigs were bioassayed in 10,000 mice and 183 hearts were bioassayed additionally in cats (the cat bioassay is known to be more sensitive when testing tissue cysts). *T. gondii* was not detectable by bioassay in mice

inoculated with a pepsin-digest of 100 g of hearts from each of 62 pigs that were shown to be infected by bioassay in cats (Dubey et al., 1995b)Dubey et al 1995). This study involved tissue cysts of *T. gondii*, but the same likely applies for oocysts, as low numbers of this life-stage are found in naturally exposed shellfish (Hohweyer et al., 2013), and oocysts may be less infective, at least for cats (Dubey, 1996).

At present, the only alternative approach to bioassays for assessing *T. gondii* oocyst infectivity is *in vivo* cell culture. In this method, samples are treated so any sporulated oocysts excyst, releasing sporozoites, which then penetrate into the cells, differentiate into tachyzoites and multiply. The presence of *T. gondii* can then be detected using PCR assays or other methods such as microscopy or IFA (Dumètre et al., 2008). Previous research has used cell culture assays to determine the effects of various treatments, including UV and irradiation on oocyst infectivity in simple matrices (Dumètre et al., 2008; Villegas et al., 2010; Ware et al., 2010). Interestingly, since the onset of this project, the technique has also been applied to shellfish tissues and other environmental samples in a laboratory setting and appears to be analytically sensitive with results correlating well with those from bioassays (Rousseau et al., 2019a). However, the work of Rousseau et al. (2019a) has not yet been applied to field samples and, although a more rapid alternative to the bioassay, the approach is technically demanding and may not always be available or appropriate for large-scale surveillance.

Several other assays have been developed which can potentially show the presence of viable *T. gondii* oocysts, including excystation (Freyre & Falcón, 2004), vital dye (Murray & Cohn, 1979; Rousseau et al., 2019b) and RT-PCR assays (Villegas et al., 2010) (reviewed in detail in Rousseau et al., 2018). Of these, RT-PCR methods seem most promising for testing large numbers of naturally exposed shellfish, as sensitive, rapid, relatively inexpensive, and potentially quantitative means for determining *T. gondii* oocyst viability where bioassays (and now cell culture tests) are not available or warranted to assess infectivity. Particularly, messenger RNA (mRNA) RT-PCR assays have emerged which target the SporoSAG gene of *T.*

gondii (Radke et al., 2004; Villegas et al., 2010; Travaillé et al., 2016). Discovered by Radke et al. (2004), the SporoSAG gene codes for a sporozoite-specific Surface Antigen Glycoprotein (SporoSAG), the dominant surface coat protein expressed on the surface of sporozoites. These assays are based on two premises: 1) that mRNA is only detectable in metabolically active, live, and therefore viable, cells; 2) that the SporoSAG gene is highly expressed only in sporulated oocysts of *T. gondii*. With regards to the latter premise, transcriptomic analysis of *T. gondii* RNA from all five developmental stages was carried out by Fritz et al. (2012) using a commercially available microarray. The authors evaluated differences in transcript abundance and found that the timing of increased expression of SporoSAG was coincident with the formation of sporozoites within the oocyst (Fritz et al., 2012). A quantitative RT-PCR targeting SporoSAG mRNA was developed and first implemented by Villegas et al. (2010) and this study confirmed that high levels of SporoSAG mRNA are detected in sporulated oocysts, whereas low levels are detected in unsporulated oocysts, and none in tachyzoites/bradyzoites. With regards to the former premise, the half-life of SporoSAG mRNA is not yet known, i.e. how long it is present after oocyst death (metabolic inactivity), but Travaillé et al. (2016) found no PCR signal from sporulated oocysts heated to 99°C for 5 min, a result recently confirmed by Rousseau et al. (2019b). SporoSAG mRNA RT-PCRs have been used to show that *T. gondii* oocysts remain viable on basil leaves after storage at 4°C for 8 days under experimental conditions (Hohweyer et al., 2016) and for evaluation of the viability and potential infectivity of *T. gondii* oocysts following various inactivation treatments (Villegas et al., 2010; Ware et al., 2010). Results of the inactivation experiments show that, depending on the treatment applied to oocysts, RT-PCR assays do not always correlate with infectivity assays (cell culture, mouse bioassay) and consequently can overestimate the number of infective oocysts (Rousseau et al., 2018). However, they can be used to detect viable, potentially infectious sporulated oocysts rapidly. Although attempts to use these RT-PCR techniques to detect viable, sporulated *T. gondii* oocysts in shellfish tissues have not yet been made, this may be a productive avenue to explore. Particularly, as Villegas et al. (2010) explain, mRNA is abundantly expressed in metabolically active, live cells, which should result in relatively low detection limits for the assays. As such these assays may be useful as a sensitive means to rapidly confirm the presence and levels of viable,

sporulated oocysts in a sample or in the environment, although unfortunately they cannot definitively show that oocysts are infectious at time of testing.

1.2.5.3.3 Applications in the New Zealand context

In New Zealand there is very limited information on *T. gondii* in the marine environment and presence of *T. gondii* has not yet been investigated in shellfish. With respect to potential shellfish species that could act as biosentinels in New Zealand, Hohweyer et al. (2013) give pointers, stating that mussels, clams, and oysters are prime target species because they are the most consumed mollusc species worldwide. Sampling a single shellfish species may be beneficial to account for species-dependent filtering activity, amongst other variables (Hohweyer et al., 2013). In New Zealand, the green-lipped mussel is an endemic shellfish species that can filter up to 9 L of seawater per hour and is found throughout coastal waters (James et al., 2001). This species lives close to shore, making them easily accessible for sampling, and also placing them at risk of contamination by protozoan cysts and oocysts that enter the marine environment in agricultural runoff, sewage discharge, and freshwater runoff. Green-lipped mussels occur naturally in Hector's and Maui dolphin habitat and these mussels are commonly harvested recreationally for human consumption, meaning that surveillance for *T. gondii* and other terrestrially derived protozoans such as *Cryptosporidium* spp. and *Giardia* spp. in this species is relevant both for marine wildlife health as well as public health. In addition, green-lipped mussels are farmed commercially in New Zealand, and it is estimated that over 63,000 metric tons of green-lipped mussels are consumed annually by New Zealanders alone (King & Lake, 2013). Although little is known regarding human toxoplasmosis in New Zealand, shellfish consumption has been identified as a significant risk factor for human toxoplasmosis in the United States (OR = 2.22, $p < 0.05$) (Jones et al., 2009) and Taiwan (OR = 3.7, $p = 0.008$) (Chiang et al., 2014). Also, consumption of recreationally sourced shellfish has been linked to two cases of giardiasis in humans reported in New Zealand (Scholes et al., 2009), further suggesting that protozoan (oo)cysts are making their way to New Zealand coastal waters and can contaminate seawater and shellfish in sufficient numbers to be a health risk. Investigation in New Zealand could therefore involve using validated molecular methods to test green-

lipped mussels for *T. gondii*, determine whether sporulated, potentially infectious oocysts are present, and to genotype any positive samples. Results should permit a better understanding of the extent of marine contamination with the parasite and assessment of genotypes circulating in nearshore coastal waters.

1.2.6 *Toxoplasma gondii* in cetaceans

Based on histological characteristics, Bandoli & de Oliveira (1977) described the first report of toxoplasmosis in a cetacean species in the world, involving a Guiana dolphin (*Sotalia guinensis*) which stranded in Brazil. Inskeep et al. (1990) provided the first report of toxoplasmosis in cetaceans published in the English language, with the discovery of fatal disseminated disease in a female Atlantic bottlenose dolphin (*Tursiops truncatus*) that stranded in Florida, USA, by histology and electron microscopy.

Toxoplasma gondii infection has since been discovered in a number of cetacean species. In some animals, infection has only been determined serologically. These include harbour porpoise (*Phocoena phocoena*) (Cabezón et al., 2004), Amazon river dolphin (*Inia geoffrensis*) (Santos et al., 2011), Black Sea bottlenose dolphin (*Tursiops truncatus ponticus*) (Alekseev et al., 2009), pantropical spotted dolphin (*Stenella attenuata*) (Obusan et al., 2015), orca (*Orcinus orca*) (Murata et al., 2004), short-beaked common dolphin (*Delphinus delphis*) (Forman et al., 2009), and humpback whale (*Megaptera novaeangliae*) (Forman et al., 2009).

Toxoplasmosis (i.e. the disease, rather than latent infection) has been diagnosed in the following odonotocete species: Atlantic bottlenose dolphin (*T. truncatus*) (Cruickshank et al., 1990; Inskeep et al., 1990; Di Guardo et al., 1995a, b; Schulman et al., 1997; Dubey et al., 2003, 2008; Cabezó et al., 2004), Atlantic spotted dolphin (*Stenella frontalis*) (Arbelo et al., 2013), striped dolphin (*Stenella coeruleoalba*) (Domingo et al., 1992; Di Guardo et al., 2011; Alba et al., 2013), Indo-Pacific bottlenose dolphin (*Tursiops*

aduncus) (Jardine & Dubey, 2002); Indo-Pacific humpback dolphin (*Sousa chinensis*) (Bowater et al., 2003), spinner dolphin (*Stenella longirostris*) (Migaki et al., 1977, 1990), Risso's dolphin (*Grampus griseus*) (Di Guardo et al., 1995a; Resendes et al., 2002), Guiana dolphin/tucuxi (*Sotalia guinensis*) (Bandoli & de Oliveira, 1977; Gonzales-Viera et al., 2013), beluga whale (*Delphinapterus griseus*) (De Guise et al., 1995; Mikaelian et al., 2000), and Hector's dolphin (*Cephalorhynchus hectori*) (Roe et al., 2013). Infection with *T. gondii* has also recently been reported in a mysticete species, the fin whale (*Balaenoptera physalus*) (Mazzariol et al., 2012).

With respect to cetacean cases of toxoplasmosis, clinical data is usually not available as animals strand dead or die soon after stranding. Thus, toxoplasmosis is mainly diagnosed at necropsy. At necropsy, gross lesions of toxoplasmosis are often absent. The most consistent gross findings in cases of disseminated toxoplasmosis are lymphadenopathy and splenomegaly (Resendes et al., 2002; Bowater et al., 2003; Miller, 2008). Other gross lesions include emaciation (Migaki et al., 1990; Bowater et al., 2003), marked congestion of meningeal vessels (Di Guardo et al., 2010), and adrenal gland congestion (Resendes et al., 2002). *Toxoplasma gondii* organisms can be demonstrated by histology and immunohistochemistry in the brain, liver, lungs, heart, spleen, and adrenals, and less frequently in the kidneys, secretory stomach, intestines, eye, optic nerve, and tonsils (Cruickshank et al., 1990; Migaki et al., 1990; Domingo et al., 1992; Di Guardo et al., 1995a, b; Mikaelian et al., 2000; Bowater et al., 2003; Van Bressem et al., 2006; Dubey et al., 2009b; Di Guardo et al., 2010; Arbelo et al., 2013; Roe et al., 2013).

Toxoplasmosis has sometimes been considered a secondary disease in cetaceans, becoming symptomatic only when animals are immunosuppressed, for example due to concurrent morbillivirus infection, or high loads of environmental pollutants (Migaki et al., 1990; Domingo et al., 1992; Mikaelian et al., 2000; Van Bressem et al., 2009; Mazzariol et al., 2012). Serological surveys of wild populations have shown that exposure to the parasite may be extremely common. For example, around 90 % of Atlantic bottlenose

dolphin (*T. truncatus*) from the coasts of Florida, South Carolina, and California, had antibodies to *T. gondii* by the modified agglutination test (MAT) (Dubey et al., 2008). Similarly, 87 % of 95 seemingly healthy Amazon River dolphin (*I. geoffrensis*) had antibodies to the parasite (Santos et al., 2011), indicating that the parasite does not necessarily cause disease. However, there is also evidence to suggest that *T. gondii* is a primary pathogen of cetaceans. Fatal disease was confirmed by immunohistochemistry of brain tissue of two striped dolphin with meningoencephalitis. *Toxoplasma gondii* cysts were closely associated with lesions in the two animals, and both were negative for morbillivirus. Likewise, a Guiana dolphin diagnosed with fatal toxoplasmosis was negative for morbillivirus and had low levels of persistent organochlorines (Gonzales-Viera et al., 2013). As well as these sporadic cases, *T. gondii* may also be a significant cause of mortality at a population level for cetaceans. Roe et al. (2013) found disseminated toxoplasmosis as a cause of death in 7/28 (25 %) of stranded Hector's dolphin. Tissue levels of polychlorinated biphenyls (PCBs) and organochlorines in Hector's dolphins tested to date were below the range believed to cause immunosuppression (Baker, 1978; Jones et al., 1999; Stockin et al., 2010), and the affected animals were negative for morbillivirus (Roe et al., 2013). In addition to causing direct mortality, *T. gondii* infections may also have indirect effects on population dynamics through behavioural changes, reproductive loss and increased risk of predation (Webster, 2001; Kreuder et al., 2003).

1.2.6.1 Transmission of *T. gondii* to cetaceans

For terrestrial animals, there are two main sources of post-natal *T. gondii* infection. These are the ingestion of oocysts in contaminated food or water and the ingestion of *T. gondii*–infected tissues of other terrestrial species. The route of infection for cetaceans, as for the sea otter, is therefore particularly interesting, as the cetacean diet primarily consists of fish or invertebrates, cold-blooded animals which do not seem to be parasitised by *T. gondii*. Although accidental ingestion of infected bird or rodent carcasses by cetaceans is possible, it is unlikely to account for the high seroprevalence of toxoplasmosis (Dubey et al., 2009b; Jones & Dubey, 2010). Cetacean infections, particularly in coastal species, are believed to be acquired through consumption of prey species and water contaminated by *T. gondii* oocysts (Lindsay et al., 2003; Miller et al.,

2002a; Miller et al., 2008; Massie et al., 2010; Marino et al., 2019). Infection and seropositivity of pelagic cetaceans is more intriguing as these animals are unlikely to come into contact with land-sea run-off. It is possible that toxoplasmosis in pelagic cetaceans such as beluga whale and striped dolphin is linked to ship run-off, when cats, or contaminated soil are onboard (Van Bressem et al., 2009), or more likely migratory fish serve as paratenic vectors. Vertical transmission of infection is also documented in cetaceans (Jardine & Dubey, 2002; Resendes et al., 2002; Roe et al., 2013).

1.2.6.2 Hector's dolphins and the importance of toxoplasmosis for the species

There are two recognised subspecies of Hector's dolphin: Māui dolphin (*C. hectori maui*) and Hector's dolphin (*C. hectori hectori*), which are divided into four genetically and geographically distinct populations. Māui dolphins inhabit the West Coast of the North Island of New Zealand, and the Hector's subspecies live in three geographically distinct groups around the South Island (Pichler et al., 1998; Pichler, 2002). Both the distribution and population size of Hector's and Māui dolphins were believed to have declined substantially as a result of fisheries-related mortality since the 1970s (Hamner et al., 2014). Although the population of South Island Hector's dolphins was recently found to be greater than previously thought, with total abundance estimated at 14,849 individuals (95 % confidence interval (CI) 11,923 – 18,492) (MacKenzie & Clement, 2016), only 63 Māui dolphins above one year of age are estimated to remain (95 % CI: 57–75) (Baker et al., 2016). The Māui subspecies is therefore considered Critically Endangered by the International Union for the Conservation of Nature (IUCN) and Nationally Critical under the New Zealand Threat Classification (Reeves et al., 2013; Baker et al., 2019). The Hector's subspecies is now considered to be Threatened – Nationally Vulnerable (Baker et al., 2019).

Both subspecies are legally protected in New Zealand and conservation efforts are jointly led by the Department of Conservation (DOC) and the Ministry for Primary Industries (MPI), guided by The Māui and Hector's Dolphin Threat Management Plan (TMP). At the onset of this project in 2015, the number one

threat against the species identified by the TMP (DOC, 2007) and associated risk assessment of threats to Māui dolphins (Currey et al., 2012), was fisheries bycatch in gill and trawl nets. TMP-related conservation efforts therefore focused management efforts on fishing restrictions. Infectious disease, however, may also be a major cause of mortality for the Hector's dolphin as a species, the impact of which has only just begun to be considered in detail with respect to threat management (Fisheries New Zealand and the Department of Conservation, 2019; Cooke et al., 2019; Roberts et al., 2019a, b).

Indeed, toxoplasmosis appears to be an important cause of mortality for these dolphins. Research conducted at Massey University, published in 2013 and recently updated in Roberts et al. (2019b), identified disseminated toxoplasmosis as the cause of death for 16 % (9/55) of Hector's dolphins (over the age of one year) necropsied between 2007 and 2018, including two (of five) Māui dolphins. For comparison, 22 % (12/55) of deaths in the case series were attributed to bycatch. Findings indicate that *T. gondii* is widespread in the coastal waters of New Zealand, as fatal toxoplasmosis cases included dolphins recovered from the east and west coasts of the South Island, as well as the west coast of the North Island (Figure 3). However, adult females may be particularly at risk of disseminated disease, and as all fatal cases occurred in dolphins recovered in spring (September, October, November) there may be a seasonality in toxoplasmosis mortality (Figure 3), although this could alternatively reflect a seasonal bias in stranding or carcass retrieval. In addition, Roe et al. (2013) found that isolates from all fatal disseminated toxoplasmosis cases were representative of one particular strain of *T. gondii*, namely variant Type II (ToxoDB #3).

Infection with *T. gondii* is also likely to have wider consequences for Hector's dolphins beyond fatal disseminated disease. Toxoplasmosis could be an important cause of neonatal loss, with the possibility of vertical transmission of the parasite from mother to calf indicated by the presence of *T. gondii* organisms in the uterine tissues of two dolphins in the study of Roe et al. 2013, as well as a case of fatal toxoplasmosis in a pregnant female that had foetal, uterine and placental tissues which were positive for *T. gondii* DNA.

Latent infections with *T. gondii* in Hector's dolphins also appear be prevalent, since ten of the dolphins assessed by Roe et al. (2013) were found to be positive for *T. gondii* DNA without evidence of active disease. Latent infections may further affect this species by altering behaviour, increasing the risk of predation, bycatch and trauma (Webster, 2001; Kreuder et al., 2003; Roe et al., 2013).

The life history of the Hector's dolphin may make them particularly vulnerable to population decline arising from threats such as toxoplasmosis. Hector's dolphins are relatively short-lived (up to 22 years) (Rayment et al., 2009) and females become sexually mature at a relatively late age (seven to nine years) (Slooten, 1991). They also have a low reproductive rate, as a female only has a single calf every two to three years (Slooten & Lad, 1991). Reduction in gene flow due to population fragmentation is also of concern, particularly for the Māui dolphin, as small isolated subpopulations are likely to be less resilient against human-induced impacts and disease (Currey et al., 2012; Baker et al., 2019). Furthermore, habitat use and foraging strategies of the Hector's dolphin may place this species at a higher risk of exposure to *T. gondii*. Survey and modelling work has shown that Hector's dolphins have a strong preference for highly turbid water and locations in which prey availability is highest (Rayment et al., 2010; Dawson et al., 2013). As such, highest dolphin densities occur in shallow nearshore waters, meaning they overlap with many human coastal activities and are in close proximity to freshwater outflows and surface runoff. As individuals, they also have a relatively small range, averaging 35 km (Currey et al., 2012; Oremus et al., 2012). Māui dolphins are primarily observed within 2 km of the coastline, and the population has a well-documented seasonal distribution (Ferreira & Roberts, 2003; Slooten et al., 2005; Oremus et al., 2012). The dolphins cluster inshore in the austral summer but are more evenly distributed in winter, a pattern thought to be driven by prey availability, as some prey species show a similar inshore distribution during summer (Beentjes et al., 2002; Rayment et al., 2010; Miller et al., 2013; Miller, 2015; Roberts et al., 2019b). Additionally, the dolphins may seek sheltered, shallow habitat during the summer calving season (Brough et al., 2019). Although Hector's dolphins consume a variety of fish and invertebrates from throughout the water column, a large proportion of their diet consists of prey from the demersal and benthic zones (bottom feeders)

(Miller et al., 2013) where *T. gondii* oocysts may accumulate in sediment and prey (Shapiro et al., 2012b; Roe et al., 2017). Stomach content analysis has shown there may be some variability in diet between populations, but red cod (*Pseudophycis bachus*), ahuru (*Auchenoceros punctatus*), and sole (*Pelotretis flavilatus*) seem to be the main constituents of the dolphin diet (Miller et al., 2013). Overall, due to their proximity to the shore, and preference for foraging in or near the benthos, combined with their late sexual maturity and slow breeding rate, the effects of terrestrially derived disease on this species may be considerable.

Since the initial discovery of toxoplasmosis in Hector's dolphins, there has been a growing scientific and political realisation that the focus of threat management on reducing bycatch is too narrow. Efforts are now underway to include toxoplasmosis in the latest reiteration of the TMP (Fisheries New Zealand and the Department of Conservation, 2019; Cooke et al., 2019; Roberts et al., 2019a, b), and the spatial risk assessment of Roberts et al. (2019b) provides important evidence to suggest that toxoplasmosis has population-level effects in this species. While the processes promoting disease in wild animal populations are highly complex, their identification is critically important for conservation when disease is limiting a population (Johnson et al., 2009). As highlighted in this chapter, there are still many unknowns and uncertainties surrounding the study of these issues in New Zealand. Further investigation into land-sea transmission of *T. gondii* in New Zealand is therefore needed, which comprises the overarching theme of this thesis.

Chapter 2 assesses the prevalence of *T. gondii* infection and toxoplasmosis in other New Zealand cetaceans, particularly the Common dolphin (*Delphinus delphis*).

Chapters 3 – 5 investigate *T. gondii* in three subgroups of New Zealand cats. Chapter 3 uses Bayesian latent class modelling to determine true seroprevalence of *T. gondii* amongst owned cats in New Zealand and to

assess the appropriateness of available serological tests in the country for feline testing. Chapter 4 estimates the burden of *T. gondii* oocysts in New Zealand through determining *T. gondii* oocyst shedding prevalence in stray and feral cats in New Zealand. The genotypes of *T. gondii* contaminating the environment in cat faeces is also investigated. Chapter 5 gives a detailed description of feline cases of toxoplasmosis in the country and assesses the genotypes of *T. gondii* associated with fatal disease in New Zealand owned cats.

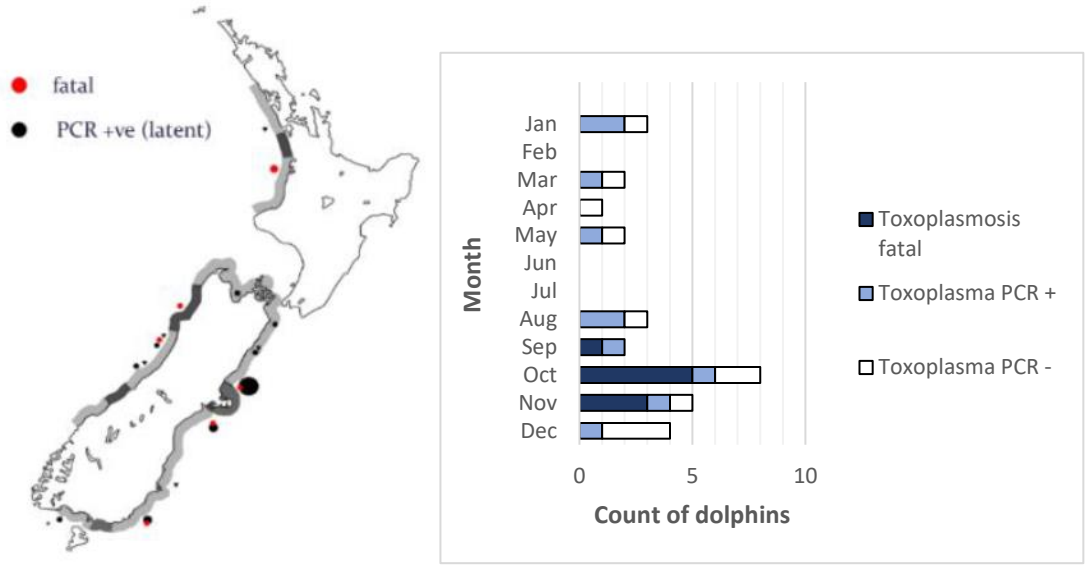
Chapter 6 investigates the prevalence of *T. gondii*, as well as other pathogenic protozoans of public health concern, namely *Cryptosporidium* spp. and *Giardia duodenalis*, in commercially sourced green-lipped mussels, as sentinels of terrestrial protozoans in marine waters in New Zealand.

Chapter 7 validates and compares four molecular methods (PCR) for *T. gondii* detection in green-lipped mussel haemolymph, to determine the best possible assay for cost-effective testing of large numbers of mussels for *T. gondii* surveillance.

Chapter 8 attempts to determine prevalence of and risk factors for *T. gondii* contamination of New Zealand coastal waters using wild green-lipped mussels as sentinels.

Chapter 9 provides a general discussion of the findings of the seven research chapters and concludes this thesis investigating *T. gondii* in the marine environment in New Zealand.

Figure 3 Spatial distribution of recovered Hector's and Māui dolphin carcasses in New Zealand (left). Red circles represent dolphins for which toxoplasmosis was determined to be the primary cause of death (fatal). Black circles represent dolphins confirmed to have been infected with *T. gondii* by PCR analysis, but which did not die from toxoplasmosis (PCR +ve (latent)). Results show that T. gondii infection and fatal toxoplasmosis is widespread in the Hector's and Māui dolphin range. Seasonal distribution of *T. gondii* infections (right), including the total number of dolphins necropsied by month from 2007 to 2018. Necropsy data was drawn from Roe et al. (2013) and Roberts et al. (2019b). Results show that all fatal toxoplasmosis cases (*n* = 9) were recovered in the austral spring (September through November), suggesting a seasonality to disease. Pale shaded coastal areas indicate regions where Hector's dolphins have been sighted. Darker shaded regions are those of the highest population density. Adapted from Roe et al. (2013) and Roberts et al. (2019b) with permission.



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2 Investigating toxoplasmosis in New Zealand dolphins

2.1 Introduction

The importance of infectious diseases for marine mammals has become increasingly apparent over the last two decades (Cole et al., 2000; Gulland & Hall, 2007; Kreuder et al., 2003; Miller et al., 2008; Roe et al., 2013; Simeone et al., 2015; Van Bressem et al., 2009). Notably, toxoplasmosis has emerged as a cause of mortality for marine mammals worldwide, including sea otters (*Enhydra lutris*), pinnipeds, sirenians, and cetaceans (e.g. Cole et al., 2000; Van Bressem et al., 2009; Dubey, 2010; Bossart et al., 2012; Roe et al., 2013; Simeone et al., 2015). The causative agent of toxoplasmosis is the protozoan parasite, *Toxoplasma gondii*. This parasite has a complex lifecycle; it is assumed that all warm-blooded animals can act as intermediate hosts of *T. gondii*, but felids are the only known definitive host in which the parasite can sexually reproduce. An infected cat can shed millions of oocysts in its faeces into the environment (Tenter et al., 2000), which may enter coastal waters via surface run-off (Miller et al., 2002; VanWormer et al., 2014, 2016). Although infection and transmission mechanisms in the marine environment are not clearly defined, evidence of *T. gondii* exposure has now been documented in numerous cetacean species globally (reviewed in Chapter 1) (Bandoli and de Oliveira, 1977; Inskeep et al., 1990; Migaki et al., 1990; Cruickshank et al., 1990; Domingo et al., 1992; De Guise et al., 1995; Di Guardo et al., 1995a, b; Schulman et al., 1997; Mikaelian et al., 2000; Jardine & Dubey, 2002; Resendes et al., 2002; Dubey et al., 2003, 2008; Bowater et al., 2003; Forman et al., 2009; Mazzariol et al., 2012; Alba et al., 2013; Arbelo et al., 2013; Gonzales-Viera et al., 2013; Herder et al., 2015; van de Velde et al., 2016; Pintore et al., 2018; Bigal et al., 2018).

Recent post-mortem investigations suggest that *T. gondii* may be especially pathogenic for the endangered Hector's dolphin (*Cephalorhynchus hectori hectori*), which only inhabits the coastal waters of New Zealand (Roe et al., 2013). Although it is not yet clear whether toxoplasmosis has population-level effects in this species, a high proportion of deaths (25 %, n = 28) due to disseminated toxoplasmosis in a case series suggests that the Hector's dolphin may be particularly susceptible to the disease (Roe et al., 2013). To date, no cases have been reported in any other cetacean species in New

Zealand waters. Serological surveys of wild cetacean populations in other geographic regions have shown that exposure to *T. gondii* may be extremely common (e.g. 100 % (*n* = 47) wild Atlantic bottlenose dolphins (*Tursiops truncatus*) in Florida, USA (Dubey et al., 2003), and 86 % (*n* = 95) free-living Amazonian river dolphins (*Inia geoffrensis*) in Brazil were seropositive for *T. gondii* (Santos et al., 2011), but also indicate that the parasite does not necessarily cause disease (Santos et al., 2011). Most *T. gondii* infections in cetaceans are likely to be chronic and asymptomatic, as is the case for the majority of intermediate hosts, in which parasite proliferation is controlled by an innate immune response (Innes, 1997; Dubey et al., 2008; Santos et al., 2011; Yarovinsky, 2014). Outside of New Zealand, mortalities in other cetacean species have been reported due to disseminated toxoplasmosis, resulting in encephalitis, pneumonia, hepatic necrosis, necrotising adrenalitis, cardiac myonecrosis, and lymphoid necrosis (Inskeep et al., 1990; Migaki et al., 1990; Di Guardo et al., 1995a, b; Mikaelian et al., 2000; Jardine & Dubey, 2002; Bowater et al., 2003; Dubey et al., 2009; Di Guardo et al., 2010; Gonzales-Viera et al., 2013; Herder et al., 2015; Bigal et al., 2018; Pintore et al., 2018). Yet, fatal cases are usually sporadic and are frequently associated with host immunosuppression, particularly due to concurrent infection with cetacean morbillivirus (CeMV) or high tissue loads of environmental pollutants (Domingo et al., 1992; Kennedy, 1998; Migaki et al., 1990; Mikaelian et al., 2000; Van Bressem et al., 2009), factors which did not appear to be associated with the Hector's dolphin cases (Roe et al., 2013). It is therefore of particular interest to determine whether Hector's dolphins are more susceptible to *T. gondii* than other New Zealand cetaceans, and to further investigate risk factors associated with *T. gondii* infection and fatal toxoplasmosis.

It is thought that there could be a genetic basis for the apparent increased susceptibility of Hector's dolphins to *T. gondii* (Roe et al., 2013), through host-related factors (Deckert-Schlüter et al., 1994; Mack et al., 1999; Suzuki, 2002; Jamieson et al., 2008) or the effects of inbreeding depression in a small population (Acevedo-Whitehouse et al., 2003; Larson et al., 2002; Miller et al., 2004; Pichler & Baker, 2000; Spielman et al., 2004). It is also possible that parasite genotype influences the severity of disease, as suspected in a variety of other species (Boothroyd & Grigg, 2002; Grigg et al., 2001; Miller et al., 2004; Parameswaran et al., 2010; Shapiro et al., 2019), particularly as variant type II (ToxoDB #3)

representative strains were identified as the cause of all fatal disseminated toxoplasmosis cases in the Hector's dolphin case series. Additionally, habitat use and foraging strategies of the Hector's dolphin may place this species at a higher risk of exposure to *T. gondii*. Hector's dolphins predominantly inhabit shallow, turbid, near-shore waters (Dawson et al., 2013; Rayment et al., 2010; Roberts et al. 2019a) and, although they consume a variety of fish and invertebrates from throughout the water column, a large proportion of their diet consists of prey from the demersal and benthic zones (bottom feeders) (Miller et al., 2013) where *T. gondii* oocysts may accumulate in sediment and prey (Shapiro et al. 2012; Roe et al., 2017).

As yet, *T. gondii* has not been identified in any other cetaceans in New Zealand, although detailed investigations have not been conducted in any species but the Hector's dolphin. The apparent absence of toxoplasmosis in non-Hector's cetacean species could be due to sampling bias or small sample size, but it is also possible that this reflects species-specific differences in exposure to the parasite and/or susceptibility to the disease. To establish whether the Hector's dolphin is unusually susceptible to *T. gondii* infection amongst New Zealand cetaceans, methods used to test Hector's dolphins should be employed to estimate the prevalence of toxoplasmosis in other species. To control for differences in *T. gondii* exposure, attempts should be made to test a species with similar feeding preferences and habitat utilisation as the Hector's dolphin. Five other delphinid species are resident in New Zealand waters, namely the short-beaked common dolphin (*Delphinus delphis*), dusky dolphin (*Lagenorhynchus obscurus*), bottlenose dolphin (*Tursiops truncatus*), orca (*Orcinus orca*), and long-finned pilot whale (*Globicephala melas*). Several more are occasionally observed, including the striped dolphin (*Stenella coeruleoalba*) (Baker 1999). None of the resident species are an exact match to the Hector's dolphin in terms of distribution, habitat, or diet, but common, dusky, and coastal bottlenose dolphins are also known to frequent nearshore waters, and consume a variety of small fish and invertebrates (Visser, 1999; Benoit-Bird et al., 2004; Markowitz et al., 2004; Vaughn et al., 2007; Würsig et al., 2007; Meynier et al., 2008; Stockin, 2008; de Castro et al., 2015). Resident orca populations also reportedly engage in benthic foraging close to shore to catch rays (Visser, 1999).

Apart from the Hector's dolphin, however, carcass recovery and tissue sampling has only routinely been carried out for one other dolphin species in New Zealand, the short-beaked common dolphin (*Delphinus delphis*) due to the instigation of the New Zealand Common Dolphin Project (NZCDP) in 2002 (Stockin et al., 2009), although other species are sporadically sampled. The aim of this study was therefore, to assess the prevalence of *T. gondii* infection in the short-beaked common dolphin, and other available delphinid species, by testing archived tissues for the presence of *T. gondii* organisms and lesions, using histology, immunohistochemistry, and polymerase chain reaction (PCR).

2.2 Methods

2.2.1 Sample collection

All tissue samples were provided by the New Zealand Common Dolphin Project (NZCDP) based under the Coastal-Marine Research Group (Massey University, Albany). As part of the NZCDP, the biology of New Zealand short-beaked common dolphins (*Delphinus delphis*) is under investigation via dissection of carcasses of dolphins that have stranded or been commercially by-caught within New Zealand waters (Stockin et al., 2009). Other delphinid species, including dusky dolphin (*Lagenorhynchus obscurus*) and striped dolphin (*Stenella coeruleoalba*), are examined when possible. Although frozen tissues are preferable for molecular diagnostic assays such as PCR (Roe et al., 2013), these were not available for the species under investigation, hence formalin-fixed paraffin-embedded (FFPE) tissues were used for this purpose. In this study, formalin-fixed tissue samples from 35 common dolphins, 7 dusky dolphins, and 2 striped dolphins examined between August 2008 and August 2015, were evaluated (N = 44). Limited gross necropsy details were also available for each animal.

2.2.2 Histopathology

For each dolphin, suitable formalin-fixed tissues were retrieved for analysis (Roe et al., 2013). In previous cetacean cases of toxoplasmosis, *T. gondii* was most frequently detected by routine histology and immunohistochemistry in the brain, liver, lungs, lymph nodes, heart, spleen, and adrenals (Migaki

et al., 1990; Cruickshank et al., 1990; Domingo et al., 1992; Di Guardo et al., 1995a, b; Mikaelian et al., 2000; Bowater et al., 2003; Van Bressem et al., 2009; Dubey et al., 2009; Di Guardo et al., 2010; Arbelo et al., 2013; Roe et al., 2013; Herder et al., 2015; Bigal et al., 2018). In this study, suitable tissues available for testing included lung, liver, and lymph node. Briefly, formalin was sieved from the sample bags, and tissues were placed on a cutting board for identification of tissue type. Selected tissues were trimmed to achieve a flat surface, approximately 2 mm in thickness. Trimmed specimens were placed in cassettes for subsequent paraffin embedding, at an angle to help with sectioning. Full cassettes were loaded into cassette holders in a formalin tub before processing for embedding. Samples were processed on a ThermoFisher® Excelsior ES Tissue Processor. The samples were dehydrated through graded alcohols (70 %, 95 % and absolute alcohol) at an ambient temperature, cleared in xylene and impregnated with Histosec® pastilles under pressure at 60 °C. The samples were then embedded using a HistoStar Embedder (ThermoFisher). Once embedded in paraffin, blocks were chilled on ice for ~ 5 min and then cut using a microTec® Rotary Microtome or Leica® RM2235 Rotary Microtome with Feather S35 disposable blades. Two sections per slide were cut at a thickness 4 µm with 30 mu distance between each slice for staining with haemotoxylin and eosin (H&E). The H&E sections were floated onto a Thermo® Tissue Bath at 43 °C and then mounted onto HDS Adhesive precleaned slides (90 ° ground edges, 76 mm x 26 mm). The slides were then stained on a Leica® Autostainer XL. The stained slides were coverslipped on a Leica® CV5030 Coverslipper using Grale HDS 24 x 50 mm #1 Cover glass. The mountant used was Entellan® manufactured by Merck. All H&E slides were examined by a veterinary pathologist.

2.2.3 Immunohistochemistry

Immunohistochemistry (IHC) for *T. gondii* was carried out on a selection of FFPE tissues from all dolphins (see Table 2), following Roe et al. (2013), and the protocol detailed in Appendix 2i. To summarise, paraffin embedded tissue sections were cut at 5 µm and mounted on positively charged glass slides (DAKO FLEX IHC Microscope Slides), then rehydrated through a series of increasing concentrations of xylene using a Leica® Autostainer XL. Proteolytic-induced epitope retrieval (PIER) was

performed in 1 % trypsin/calcium chloride solution (pH 7.8) for 30 min at 37 °C. Endogenous peroxidase activity was blocked by incubating slides in 0.3 % hydrogen peroxide for 30 min at room temperature, followed by washing in 50 mM tris-buffered saline (TBS). Primary antibody (polyclonal caprine anti-*T. gondii* antibody; VMRD Inc., Pullman, WA, USA) (150 µl) diluted to 1:1000 in 0.1 % gelatin/TBS was then applied to the slides which were kept refrigerated at 4 °C overnight. Slides were then washed with TBS before incubation for 30 min at room temperature in 150 µl biotinated donkey origin anti-goat IgG secondary antibody (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) diluted to 1:200. ABC reagent (Vectastain Universal Elite kit, Vector Laboratories, Burlingame, California, USA) was then applied and the resulting complex was visualised using 3-3' - diaminobenzidine (DAB) diluted in distilled water (DAB Peroxidase Substrate Kit, Vector Laboratories). Sections were counterstained in haematoxylin and mounted. Positive antibody controls were run with each batch of slides using sequence-confirmed *T. gondii* positive cat lung (45286-15) and Hector's dolphin tissue sections (45670-4). Negative controls comprised the omission of anti-*T. gondii* primary antibody on known positive cat and dolphin sections.

2.2.4 DNA extraction and polymerase chain reaction (PCR)

FFPE blocks were sectioned at 10 µm for DNA extraction using a new blade and cleaning the microtome with Fuelite between blocks. Forceps were also kept in a beaker of 70 % ethanol to limit cross-contamination. DNA was extracted from all selected FFPE specimens using DNeasy Blood and Tissue kits (Qiagen, Germany), following manufacturer's protocols for pre-treatment for paraffin embedded tissue, and purification of total DNA from animal tissues (spin-column), except that samples were left to incubate with proteinase K at 56 °C overnight, and DNA was eluted in 100 µl AE buffer. Water blanks were included as sample processing controls to confirm lack of contamination during the extraction process. DNA was kept at -20 °C until further analysis was performed. Primers used in subsequent PCR reactions are summarised in Table 1. A nested PCR (nPCR) assay targeting a ~450 bp fragment of the *T. gondii dhps* gene (dihydropteroate synthase) was used to detect *T. gondii* DNA (Aspinall et al., 2002, Appendix 2ii). Primary PCR reaction mixtures (25 µl final volume) included 10X PCR Buffer (2.5 µl), 50 mM $MgCl_2$ (0.75 µl), 10 mM dNTP mixture (0.5 µl), Platinum® Taq polymerase (0.2 µl), 10 µM FOOD1

(0.5 µl), 10 µM FOOD2 (0.5 µl), and 2.5 µl extracted DNA as the template. Secondary PCR reaction mixtures (50 µl final volume) included 10X PCR Buffer (5.0 µl), 50 mM $MgCl_2$ (1.5 µl), 10 mM dNTP mixture (1.0 µl), 10 µM FOOD3 (1.0 µl), 10 µM FOOD4 (1.0 µl), Platinum® Taq polymerase (0.2 µl) (Invitrogen, California, USA), and 2.0 µl primary amplified product as the template. Amplification was performed in a Veriti 96 Well Thermal Cycler (Applied Biosystems, California, USA). Cycling conditions were as follows: for the primary reaction 94 °C for 5 min (1 cycle); 94 °C for 30 sec, 57 °C for 30 sec, and 72 °C for 1 min (40 cycles); 72 °C for 10 min (1 cycle). For the secondary PCR, 94 °C for 5 min (1 cycle); 94 °C for 30 sec, 57 °C for 30 sec, and 72 °C for 30 sec; 72 °C for 10 min. All nPCR reactions included positive and negative controls. DNA extracted from a known *T. gondii* isolate (incomplete strain S48, Toxovax®, MSD Animal Health, New Zealand), confirmed by sequencing, was used as the template for positive controls. Ultrapure water was used in no template controls (NTC). To confirm successful amplification, 10 µl of each final PCR product was run on a 1.5 % agarose gel containing SYBR Safe (Thermo Fisher Scientific, Massachusetts, USA) at 100 V for 45 min.

To confirm DNA quality and successful extraction from tissue samples, a ~111 bp fragment of the dolphin species-specific housekeeping gene (GAPDH) was amplified from all lung samples using a technique adapted from Spinsanti et al. (2006), following Buckle et al. (2017), and as detailed in Appendix 2iii (Table 1). A 50 µl PCR mixture contained 10X PCR Buffer (5.0 µl), 50 mM $MgCl_2$ (1.5 µl), 10 mM dNTP mixture (1.0 µl), 10 µM GAPDH-F (1.0 µl), 10 µM GAPDH-R (1.0 µl), Platinum® Taq polymerase (0.2 µl), and 5.0 µl (10 ng) DNA. The PCR was conducted as above, with minor modifications. Cycling parameters here were 94 °C for 5 min (1 cycle); 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 1 min (40 cycles); 72 °C for 10 min (1 cycle). The GAPDH positive control used was DNA extracted from well-preserved Hector's dolphin tissue. To confirm successful amplification, 10 µl of each final PCR product was run on a 1.5 % agarose gel containing SYBR Safe (Thermo Fisher Scientific) at 100 V for 45 min. As individual bands were too weak for direct sequencing, PCR products with the brightest bands were pooled for each dolphin species (70 µl total volume) and run on a 1.5 % agarose gel before gel purification. Bands were purified using Qiaex II Gel Extraction Kit (Qiagen), following manufacturer's instructions. Quality of the purified product was assessed by running 5 µl of the

elution, diluted with 5 µl nuclease-free water, on a 1.5 % agarose gel. Samples were then submitted to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems) using forward and reverse primers. Sequences were analysed using Geneious software 8.1.5 (Biomatters, New Zealand) and compared with reference sequences for GAPDH using a BLAST search through the GenBank database, NCBI (Benson et al., 2007).

As DNA quality may have been compromised due to prolonged fixation of tissues, dolphin DNA samples were re-tested using a real-time PCR (qPCR), which targeted a smaller fragment of the *T. gondii* genome - 81 bp of the 529 bp repetitive element (rep529) (Homan et al., 2000; Table 1; Appendix 2iv and Chapter 7). Final reaction mixtures (20 µl total) included 10 µl 2X FastStart Universal SYBR Green Master (ROX) (Sigma Aldrich, Missouri, USA), 0.25 µM forward primer, 0.25 µM reverse primer, and 2 µl of template DNA. Amplification was performed in a Mic qPCR cycler (Bio Molecular Systems, Queensland, Australia). Amplification conditions were 95 °C 15 min followed by 40 cycles at 95 °C for 15 s, 54 °C for 30 sec, and 72 °C for 30 sec. Each reaction was followed by a melting curve 70 to 85 °C, 0.1 °C per sec. DNA extracted from a known *T. gondii* isolate (as above) was used as the positive control and ultrapure water was used in the no template control (NTC).

Presence or absence of *T. gondii* infection in each dolphin was determined by considering combined results from histological/IHC and PCR analyses in parallel and used to estimate the proportion of infected animals. Confidence intervals (95 %) were calculated using the Wilson method for binomial proportion data (Newcombe, 1998) in R version 3.4.2 (R Core Development Team, 2017), using the binom v 1.1-1 (Dorai-Raj, 2014) package. Results from histological, IHC, and PCR analyses were also used to distinguish between latent, active, and fatal *T. gondii* infections where possible. Definitions of latent, fatal, and active infections used were as follows: 'latent' – tissue(s) PCR positive for *T. gondii* DNA with few to no *T. gondii* tissue cysts observed histologically with no associated inflammation and an absence of free tachyzoites (Montoya, 2002); 'fatal' - tissue(s) PCR positive for *T. gondii* DNA,

widespread lesions characteristic of toxoplasmosis observed histologically with inflammation and tachyzoites within lesions, likely disseminated infection affecting multiple tissues; 'active' - tissue(s) PCR positive for *T. gondii* DNA, tissue cysts and tachyzoites with inflammation observed histologically in one or more tissues.

Fisher Exact tests were used to assess whether the proportions of dolphins either diagnosed with fatal toxoplasmosis or with evidence of *T. gondii* infection in this study were significantly different from the proportions of Hector's dolphins observed in the study of Roe et al. (2013). Statistical analyses were considered significant when $p \leq 0.05$ and were conducted in R. The following equation was also used in this study:

$$n = \ln(\alpha)/\ln(1-p) \quad [1]$$

where α = 1 – confidence level, p = minimum prevalence (Dohoo et al., 2013)

This equation can be used to calculate the sample size needed to detect disease in an infinite population (> 1000) when it is present at or above a biologically relevant prevalence, as defined by the researcher (Fosgate, 2009).

Table 1 Primer sequences, annealing temperatures, and expected amplicon size for polymerase chain reaction assays used to amplify the *dhps* gene and 529 bp repetitive element (rep529) of *Toxoplasma gondii*, and the *Stenella* spp. GAPDH gene, from dolphin tissues

PCR Type	Gene Target	Primer Name	Primer Sequence 5' – 3'	Annealing Temp (^{o}C)	Size (bp)	Reference
nPCR	*dhps*	FOOD1	GGA ACA TCC GCT GAA GCT CAT GG	57	491	Aspinall et al., (2002)
		FOOD2	CAG AGA ATC CAG TTG TTT CGA GG			
		FOOD3	CAG TCC AGA CTC GTT CAC CGA TC	57	416	
		FOOD4	CCG GAA TAG TGA TAT ACT TGT AG			
PCR	GAPDH	GAPDH-F	CAA GGC TGT GGG CAA GGT CAT C	60	111	Spinsanti et al., (2006)
		GAPDH-R	TTC TCC AGG CGG CAG GTC AG			
qPCR	rep529	ToxoRE_f	CAC AGA AGG GAC AGA AGT CG	54	81	Kasper et al., (2009)
		ToxoRE_r	CAG TCC TGA TAT CTC TCC TCC AAG			

2.3 Results

Archived tissues were provided for 44 dolphins in total: 35 *D. delphis*, 7 *L. obscura*, and 2 *S. coeruleoalba*. The complete set of tissues (lung, liver, lymph node) was not available for all individuals, and tissue availability by species is summarised in Table 2. Formalin fixation time for each set of common dolphin tissues was calculated as the number of days between tissue collection and trimming, as tissues had been stored in 10 % neutral-buffered formalin throughout this period. Fixation times ranged from 272 to 1799 days, with a mean of 787 days (2.16 years) (Appendix 2v).

Histological lesions consistent with toxoplasmosis (multifocal haemorrhage and necrosis) were present in 1/44 dolphins (2 %). This animal was a common dolphin, with lesions present in both the liver and lung sections. No tachyzoites or tissue cysts were observed on any of the H&E-stained sections examined for any dolphin, however, and all samples were negative for *T. gondii* using IHC (total of 87 tissues from 43 dolphins). All samples (a total of 105 tissues from 43 dolphins) (see Table 2) were negative for *T. gondii* DNA using nPCR targeting the *dhps* gene. The quality of DNA extracted from dolphin tissues was assessed by amplifying the GAPDH gene, as an endogenous external amplification control. Bands of expected size were seen for 18/36 (50 %) lung tissue samples tested (14/28 (50 %) common dolphin; 4/6 (67 %) striped dolphin; 0/2 (0 %) dusky dolphin), however individual bands were too weak for direct sequencing. Positive amplicons were therefore pooled by dolphin species. Sequencing was only successful for the common dolphin pool, which despite substantial electropherogram background noise, was confirmed by NCBI BLAST to have 92 % nucleotide similarity to the GAPDH gene of striped dolphin Stenella coeruleoalba (GenBank DQ404538.1; Spinsanti et al. 2006). Following this, DNA from all dolphin lung samples that showed GAPDH amplification was re-tested for presence of *T. gondii* DNA using a rep529 qPCR targeting a smaller 81 bp fragment of the *T. gondii* genome. Amplification was seen from three samples but melt curve analysis showed that it was non-specific, meaning all lung samples were negative for *T. gondii* using this assay.

Considering all results from histological, IHC, and PCR analyses, 0/44 (0 %, 95 % CI: 0 – 8.0) of the dolphins included in the study were diagnosed with *T. gondii* infection and thus 0 % were given a diagnosis of fatal toxoplasmosis. Results by species are given in Table 2. Overall, a significant difference in the proportions of dolphins found to be infected with *T. gondii* or diagnosed with fatal toxoplasmosis was observed between animals in this study and Hector's dolphins in Roe et al. (2013) ($p \leq 0.0008$).

Table 2 Summary of PCR and IHC results

Species	**Tissue**	**No. Samples**	**PCR *T. gondii* Positive/No. Tested**	**IHC *T. gondii* Positive/No. Tested**	**Proportion overall positive (95 % CI)**
Delphinus delphis (Common dolphin) *n* = 35	Lung	34	0/34	0/34	0 (0 – 9.9)
	Liver	31	0/31	0/31	
	Lymph Node	24	0/24	0/24	
Lagenorhynchus obscurus (Dusky dolphin) *n* = 7	Lung	6	0/6	0/1	0 (0 – 35.4)
	Liver	6	0/6	0/2	
	Lymph Node	4	0/4	0/1	
Stenella coeruleoalba (Striped dolphin) *n* = 2	Lung	2	0/2	0/2	0 (0 – 65.8)
	Liver	2	0/2	0/2	
	Lymph Node	2	0/2	0/2	
Total *N* = 44					0 (0 – 8.0)

2.4 Discussion

In this study, archived tissues from stranded or bycaught New Zealand cetaceans were examined for *T. gondii* presence using histological, immunohistochemical and molecular methods. In total, tissues from 35 common dolphins, 7 dusky dolphins, and 2 striped dolphins (Aug 2008 – Aug 2015), were evaluated, but 0/44 (0 %, 95 % CI: 0 – 8) of the dolphins were found to be infected with *T. gondii*. This was a somewhat unexpected result, particularly because the presence of *T. gondii* DNA was detected by PCR in 17/28 (61 %) Hector's dolphins that stranded or were bycaught along the New Zealand coastline between 2007 and 2011 (Roe et al. 2013). Disseminated toxoplasmosis was diagnosed as the cause of death for seven (25 %) of these animals, providing evidence that *T. gondii* is present in New Zealand coastal waters, and that toxoplasmosis may be an important disease of New Zealand cetaceans. The proportions of dolphins diagnosed with *T. gondii* infection or fatal toxoplasmosis in this study were significantly lower than for Hector's dolphins ($p \leq 0.0008$). A number of reasons may be offered to explain why *T. gondii* infection was not identified in the dolphins included in this study. Whilst results may be due to issues with specimen preservation and sample size, they could also be true negatives, reflecting differences in exposure to *T. gondii* or susceptibility to toxoplasmosis between the cetacean species investigated here and the Hector's dolphin, in New Zealand.

The main purpose of formalin fixation and paraffin embedding of tissues collected at postmortem is to allow for histopathological analysis. The FFPE tissues that are generated by this process can be archived and used for subsequent histological, immunohistochemical and molecular analysis. However, it is important to note that fixation in formalin can degrade nucleic acids, resulting in extraction of DNA fragments < 300 bp, and can inhibit subsequent PCRs (Ben-Ezra et al., 1991; Masuda et al., 1999; Srinivasan et al., 2002; Bonin et al., 2003; Ferrer et al., 2007; Gilbert et al., 2007; Zimmermann et al., 2008; Turashvili et al., 2012; Dietrich et al., 2013; Granato et al., 2014; Howat & Wilson, 2014). The duration of fixation is one of the most important factors impacting the extent of DNA degradation (Ferrer et al., 2007; Dietrich et al., 2013). No standardised minimum fixation time has been agreed upon in the veterinary or medical literature, but a general rule of thumb is 1 hour per mm tissue, and a fixation time of 24 hours is recommended for neutral buffered formalin-fixed medical specimens

(Turashvili et al. 2012; Howat and Wilson 2014). Tissues are often fixed for longer periods to ensure good preservation for histopathology, but a longer duration of fixation can adversely affect the quality of tissue DNA, decrease the average size of extracted DNA fragments, and lead to ineffective target amplification in a PCR (Greer et al., 1991; Douglas & Rogers, 1998; Srinivasan et al., 2002; Ferrer et al., 2007; Dietrich et al., 2013). In cetacean studies, these problems may be compounded by the fact that many carcasses are retrieved after stranding events, where there may be some time between death and carcass recovery, so tissue quality may already be compromised (van de Velde et al., 2016). In this study, tissues were fixed in 10 % neutral buffered formalin for an average of 787 days (2.16 years; range 272 to 1799 days) before they were trimmed and embedded in paraffin and used for DNA extraction. It is therefore possible that DNA integrity from tissues in this study was not optimal for robust PCR amplification of larger targets.

A PCR amplifying a ~111 bp fragment of the dolphin species-specific housekeeping gene (GAPDH) was run as an external extraction control and to assess DNA quality (Buckle et al., 2017). Lung tissue was chosen for this purpose because it was most widely available (Table 2). The GAPDH sequence from pooled common dolphin amplicons was confirmed to be of dolphin origin. The sequence data did, however, contain substantial background noise, indicating that the DNA from the lung samples may be degraded. All available dolphin tissues were tested for *T. gondii* DNA using the nPCR *dhps* but were negative. This assay targets a ~450 bp fragment of the *T. gondii pppk-dhps* gene. As DNA quality may be suboptimal in samples from FFPE blocks produced several months to years after fixation, particularly for larger DNA fragments (Ferrer et al. 2007), it is possible the negative results obtained using the nPCR *dhps* were false negatives. To account for this, recognising that altering the size of the target amplicon in a PCR can help compensate for degradation (Iwamoto et al., 1996; Lewis et al., 2001; Bonin et al., 2003; von Ahlfen et al., 2007; Taga et al., 2013), a real-time PCR targeting an 81 bp fragment of the 529 repetitive element of *T. gondii* (rep529 qPCR) was subsequently employed. Again, all samples tested were negative for *T. gondii* DNA. These results were consistent with the histological and immunohistochemical findings, which did not demonstrate the presence of *T. gondii* in any of the dolphins examined and substantiates the negative PCR results as true negatives.

The small sample sizes available in this study mean that the possibility that toxoplasmosis is present but not detected in non-Hector's cetaceans, cannot be ruled out, however. Re-arranging equation [1], $p = 1 - exp\left(\frac{ln(\alpha)}{n}\right)$, the sample size used in this study (*n* = 44) was sufficient to find *T. gondii* if it was present in ≥ 6.6 % of the population, meaning that the prevalence of *T. gondii* in the population could have been as high as 6.6 % (with 95 % confidence) despite no detection in 44 samples (Dohoo, 2013). Considering common dolphins alone, 0 % (*n* = 35) were found to be infected, but at this sample size the population prevalence could have been as high as 8.2 %, with 95 % confidence, despite no detects (noting that these calculations do assume that the diagnostic tests were perfectly accurate and that samples were selected at random) (Dohoo, 2013).

Although there have been no fatal toxoplasmosis cases reported, seroprevalence studies conducted overseas suggest that *T. gondii* exposure is widespread in European common dolphin populations, up to 56 % (*n* = 9) in Scotland (van de Velde et al., 2016), 29 % (*n* = 21) in Britain (Forman et al., 2009), and 50 % (*n* = 4) in Spain (Cabezón et al., 2004). There have been no reports of *T. gondii* infection in dusky dolphins overseas to date, but *T. gondii* is considered a pathogen of concern for striped dolphins (Di Guardo & Mazzariol, 2013). Seven wild striped dolphins from Italy were found to be positive for *T. gondii* by PCR testing of brain, heart, lung, and melon (Giorda et al., 2017) and in an earlier Italian study, seroprevalence was estimated to be 33 % (*n* = 27) (Profeta et al., 2015). Several fatal cases of toxoplasmosis have also been reported in wild striped dolphins in Europe (Domingo et al., 1992; Di Guardo et al., 1995a, b; Cabezón et al., 2004; Alba et al., 2013; Grattarola et al., 2016). Although geographic location is thought to be an important risk factor for *T. gondii* infection (Shapiro et al., 2012), overseas studies show that common and striped dolphins are susceptible to *T. gondii*, as are most warm-blooded animals (Tenter et al., 2000). It is therefore not possible to definitively conclude that common, dusky, and striped dolphins in New Zealand are toxoplasmosis free (free from latent, active, or fatal infections), although the individual dolphins included in this study may indeed be negative for *T. gondii*. It does, however, seem likely that the prevalence of *T. gondii* infection, at least in the New Zealand common dolphin population, is lower than that in the Hector's dolphin. Taken together with the findings of this study, results add weight to the hypothesis that toxoplasmosis is

unusually prevalent in Hector's dolphins, which may reflect differences in susceptibility between host species and/or differences in exposure to *T. gondii* oocysts.

It is thought that cetaceans are mainly infected with *T. gondii* through prey ingestion or accidental consumption of water contaminated with oocysts, which enter coastal waters in surface run-off polluted with cat faeces (Miller et al., 2008), where they can survive for months to years (Dubey, 1998; Frenkel et al., 1975; Lindsay et al., 2003; Lindsay & Dubey, 2009). Surface properties of *T. gondii* oocysts mean that locations where contaminated freshwater runoff mixes with seawater may be high-risk sites for exposure (Shapiro et al., 2010). In these locations, oocysts can clump together in marine aggregates, settle, and concentrate in benthic sediment (Shapiro et al., 2010, 2012). Furthermore, oocysts can attach to biofilms and have been shown to accumulate in filter-feeding fish (Massie et al., 2010; Marino et al., 2019) and invertebrate species, such as mussels and clams (Arkush et al., 2003; Lindsay et al., 2004; Coupe et al., 2018), as well as kelp-grazing turban snails (Krusor et al., 2015), which may vector the parasite to higher trophic animals (Shapiro et al., 2019a). Differences in exposure to *T. gondii* oocysts between cetaceans in New Zealand may therefore be explained by variation in range use, habitat use, and feeding ecology, risk factors for protozoal exposure for the Southern sea otter in California (Johnson et al., 2009; Tinker et al., 2019), another marine mammal species for which *T. gondii* appears to be especially pathogenic.

Hector's dolphins predominantly live in shallow nearshore habitats (Slooten et al., 2006; Rayment et al., 2010; Dawson et al., 2013; Weir & Sagnol, 2015), particularly during summer months. They spend substantial amounts of time in harbours and are frequently found near the mouths of rivers (Weir & Sagnol, 2015). Also, although Hector's dolphins have been shown to forage throughout water column, they mainly subsist on demersal and benthopelagic (benthic) prey (Miller et al., 2013). Benthic foraging behaviour in shallow nearshore waters may increase the risk of *T. gondii* infection both through direct ingestion of filter-feeding benthic prey species and accidental ingestion of oocyst-contaminated sediment during prey capture (Michael et al., 2016). In contrast, dusky dolphins reportedly forage

offshore in deep water at night and move into shallow nearshore waters during the day to rest and socialise (Würsig et al., 1991; Markowitz, 2012). Striped dolphins are only occasionally observed in New Zealand waters but are predominantly an oceanic offshore species, found close to shore only in places where the water is deep (Van Waerebeek et al., 1998; Di Guardo et al., 2011). In addition, the main prey species in the diet of Hector's, common, and dusky dolphins in New Zealand appear to differ (summarised in Table 3), although these dolphins do share filter-feeding prey items, including lantern-fish (*Diaphus* spp.) and jack mackerel (*Trachurus* spp.) (Meynier et al., 2008); no dietary data for striped dolphins in New Zealand are available. It is also possible that differences in dolphin distributions affect exposure risk (see Figure 4). Sea otter research suggests that there may be distinct geographic zones where otters are at greater risk of infection from terrestrial pathogens, including *T. gondii*, due to a spatial combination of contaminated land-based runoff sources with distribution of contaminated prey species (Shapiro et al., 2012). In New Zealand, there may be geographic zones specific to Hector's dolphins that are hotspots of contamination. As Hector's dolphins have relatively small alongshore home ranges (ca. 50 km) and show high site-fidelity (Bräger et al., 2002; Dawson et al., 2013; Rayment et al., 2009), this could place them at greater risk of exposure.

Interestingly, on a global scale, fatal cases of toxoplasmosis in cetaceans are most often reported in wild striped dolphins after Hector's dolphins, even though striped dolphins are an "offshore" species (Di Guardo et al., 2011), highlighting the complex nature of *T. gondii* transmission mechanisms and epidemiology in marine ecosystems. A possible explanation is that there are high-risk prey items consumed by striped dolphins, such as migratory filter-feeding anchovies or sardines, which accumulate and transfer *T. gondii* oocysts from nearshore to pelagic environments (Massie et al., 2010; Di Guardo et al., 2011), although these fish are consumed by a wide variety of marine mammals (Massie et al., 2010), including common and dusky dolphins in New Zealand (Table 3).

It is also important to consider that differences in exposure to *T. gondii* oocysts between the cetacean species in this study could be due to timing of sampling, as only 14/35 (40 %) of the common dolphins

included the present study stranded or were bycaught during the same period as the Hector's dolphin case series (2007 – 2011). Risk factors for marine contamination with *T. gondii* oocysts in New Zealand have not yet been elucidated and the extent of inter-annual variability is not known. However, oocyst contamination of marine waters likely occurred in all years, both in this study and that of Roe et al. (2013), particularly as the New Zealand cat population is large and has grown (Zito et al., 2019), and coastal development has increased over the years (Collins & Kearns, 2010); higher levels of coastal development and larger domestic cat populations have previously been linked to higher levels of oocyst runoff overseas (VanWormer et al., 2016). Furthermore, all Hector's dolphins diagnosed with fatal toxoplasmosis in the case series of Roe et al. (2013) were recovered in spring (Sept, Oct, Nov), and 5/7 were adult females. In this study, only 5/35 common dolphins were recovered in spring and of these, only one dolphin was an adult female. In contrast, 23/35 of the common dolphins were found in the winter (Jun, Jul, Aug). It is possible that this seasonal disparity was due to some form of carcass recovery bias, for example, if common dolphin carcasses are more frequently beachcast in winter than spring, relative to Hector's dolphins. If so, this could have resulted in under-estimation of *T. gondii* prevalence in the common dolphins sampled. However, the disparity may also reflect a true difference in stranding patterns between the species as a result of differences in exposure and/or susceptibility to toxoplasmosis.

In addition to differences in exposure, it is thought that there could be a genetic basis for the apparent increased susceptibility of Hector's dolphins to *T. gondii* (Roe et al., 2013). Host-related factors such as major histocompatibility complex (MHC) haplotype and other genetic polymorphisms are believed to play an important role in determining resistance/susceptibility to toxoplasmosis in certain populations or individuals (Deckert-Schlüter et al., 1994; Mack et al., 1999; Suzuki, 2002; Jamieson et al., 2008). For example, variation in the gene encoding natural resistance-associated macrophage protein 1 (Nramp1/SLC11A1) is documented to influence susceptibility to toxoplasmosis and other infectious diseases in mice, as well as an array of infectious and autoimmune diseases in humans (Blackwell et al., 2001; Acevedo-Whitehouse & Cunningham, 2006; Li et al., 2011). Inbreeding depression could also be a contributing factor (Pichler & Baker, 2000; Acevedo-Whitehouse et al., 2003; Spielman et al., 2004),

as proposed for the Southern sea otter (Larson et al., 2002; Miller et al., 2004), for which toxoplasmosis is accepted as a significant cause of mortality (Kreuder et al., 2003). The Hector's dolphin consists of two subspecies: the Māui dolphin (*Cephalorhynchus hectori maui*) and the South Island (SI) Hector's dolphin (*Cephalorhynchus hectori hectori*), listed by the IUCN as critically endangered and endangered, respectively (Reeves et al., 2013). There are an estimated 14,849 (95 % CL 11,923 – 18,492) SI Hector's dolphins, found around the South Island of New Zealand in at least three genetically and geographically distinct groups (Hamner et al., 2012). The Māui dolphin is limited to the northwest coast of the North Island, with a remnant distribution of approximately 140 km (Oremus et al., 2012), and a population size estimated in 2016 at just 63 individuals over the age of 1 year (95 % CL 95 – 75; Baker et al., 2016). Due to a lack of gene flow between groups and reduced effective population size, it is therefore possible that the Hector's dolphin, particularly the Māui dolphin subspecies, suffers from genetic drift and inbreeding depression. The subsequent loss of genetic diversity may result in an increasing vulnerability to infectious diseases such as toxoplasmosis (Pichler & Baker, 2000; Acevedo-Whitehouse et al., 2003; Spielman et al., 2004).

It is also possible that certain genotypes of *T. gondii* are particularly pathogenic for Hector's dolphins but not for other cetacean species in New Zealand, as variant type II (ToxoDB #3) representative strains were identified as the cause of all fatal disseminated toxoplasmosis cases in the Hector's dolphin case series (2007 – 2011) (Roe et al., 2013). Species-specific pathogenicity of *T. gondii* strains has been documented previously in mice (reviewed in Innes, 1997) and sea otters (Shapiro et al., 2019b), although this aspect of host-parasite-environment interactions is highly complex and particularly difficult to investigate beyond laboratory animals (Dardé, 2008). Furthermore, variant Type II was more recently identified as the causal genotype of systemic toxoplasmosis in a New Zealand sea lion (Roe et al., 2017), suggesting that this strain may be widespread in the country and can be pathogenic for other marine mammal species.

Table 3 Habitat and Prey differences between dolphin species in New Zealand

Dolphin species	Main Habitat	Main Prey Species	References
Hector's dolphin	Coastal, shallow inshore waters	red cod, ahuru, Hector's lanternfish, sole, arrow squid, stargazer	Rayment et al., 2010; Miller et al., 2013; Roe et al., 2013; McKensie & Clement, 2014; Brough et al., 2019
Common dolphin	Coastal and offshore waters, daily and seasonal movement	arrow squid, jack mackerel, anchovy	Meynier et al., 2008; Stockin, 2008
Dusky dolphin	Coastal and offshore waters	pilchard, anchovy, garfish, yellow-eyed mullet, sprat	Benoit-Bird et al., 2004; Vaughn et al., 2007
Striped dolphin	Offshore waters (but see Spitz et al., 2006)[a]	NA[b]	Spitz et al., 2006; Di Guardo et al., 2010

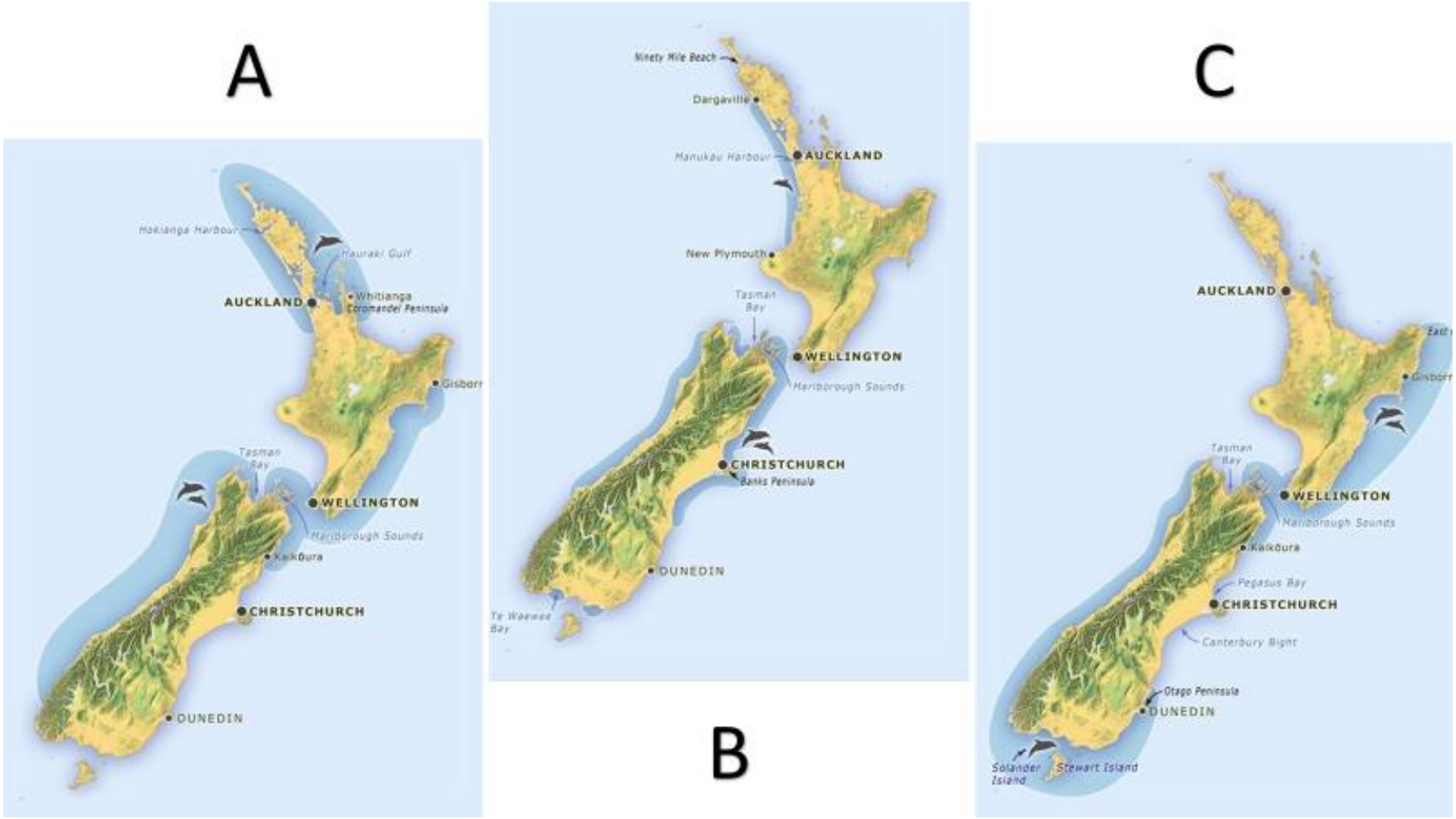
[a] Information from European striped dolphin studies. [b] NA – New Zealand specific data not yet available

In conclusion, results indicate that the dolphins included in this study were not infected with *T. gondii*, as there was no histological, immunohistochemical or molecular evidence of *T. gondii* presence. Common, dusky, and striped dolphins in New Zealand therefore appear to have a lower prevalence of *T. gondii* infection and disease than Hector's dolphins. Further research is needed, however, to confirm these findings due to methodological differences in tissue sampling between Hector's dolphins and the cetacean species included in this study and several recommendations can be made.

Investigations on toxoplasmosis in non-Hector's dolphins should include a full postmortem for results to be comparable to the Hector's dolphin study of Roe et al. (2013). Two sets of tissues should be sampled at post-mortem – a) representative samples from all major organs including brain, heart, lymph nodes, skeletal muscle, and liver should be fixed with 10% buffered formalin for histological analyses, b) to use PCR to confirm *T. gondii* infection with a high degree of certainty, representative, standardised samples from liver, lung, lymph nodes, and brain should be flash frozen and stored, preferably at -80°C (Roe et al., 2013; Herder et al., 2015; Giorda et al., 2017; Pintore et al., 2018), or maintained in a DNA/RNA preservation solution (Casale et al., 2010). Comparison of dolphins stranding over the same time frame would be ideal, as there may be a temporal component to marine contamination with *T. gondii* and thus dolphin exposure and infection. Assuming the prevalence of *T. gondii* infection in Hector's dolphins to be 61 % (17/28, Roe et al., 2013), and the prevalence of infection in non-Hector's dolphins to be 8 % (this study, upper limit of 95 % CI), a sample size of 30 dolphins per species would be required to determine a significant difference in proportions positive with 95 % certainty (Sergeant, 2018). Alternatively, assuming that the prevalence of fatal toxoplasmosis in Hector's dolphins is 25 % (7/28, Roe et al. 2013) and that fatal toxoplasmosis in non-Hector's dolphins is comparatively rare (≤ 1 %), a sample size of 76 dolphins per species would be required (Sergeant, 2018). To control for differences in *T. gondii* exposure, species with similar feeding preferences and habitat utilisation as the Hector's dolphin should be prioritised, although sample size requirements should be kept in mind. The coastal bottlenose dolphin seems to be most like the Hector's dolphin, with a nearshore coastal distribution, living in three genetically and geographically distinct groups around New Zealand (Lusseau, 2005). Little is known about coastal bottlenose feeding

ecology, however, and the population is small, so other more abundant and well-known species like the common dolphin should also be included. Orca may also be an interesting species to investigate as they have been observed close to shore engaging in benthic foraging for rays, which seems to be a behaviour unique to New Zealand populations (Visser, 1999). Investigation of *T. gondii* accumulation and survival in dolphin prey species should also be priority for future research, particularly in prey items specific to Hector's dolphins, as well as items common to the different dolphin species included, and migratory fish such as anchovies.

Figure 4 Maps of New Zealand showing the distribution of A) common dolphins (*Delphinus delphis*), B) Hector's dolphins (*Cephalorhyncus hectori*), and C) dusky dolphins (*Lagenorhynchus obscurus*) (Hutching, 2015a, b, c)



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3 *Toxoplasma gondii* in New Zealand companion cats: evaluation of serological tests, seroprevalence, and risk factors

3.1 Introduction

Toxoplasma gondii is a ubiquitous protozoan parasite that causes toxoplasmosis in humans and other warm-blooded animals, including marine mammals (Dubey, 2016). The parasite has a complex lifecycle; all warm-blooded animals may be intermediate hosts but cats are the only known definitive host of *T. gondii* and play a key role in the spread of toxoplasmosis by shedding environmentally-resistant oocysts in their faeces (Frenkel et al., 1970; Davis & Dubey, 1995; Dubey et al., 1995; Dubey, 2016), which are an important source of infection for many intermediate hosts (Dubey, 2004; Innes et al., 2009; Afonso et al., 2010; Dabritz & Conrad, 2010). Domestic cats (*Felis catus*) are mainly thought to become infected with *T. gondii* after consuming tissue cysts in prey, such as small mammals and birds (Davis & Dubey, 1995; Dubey, 2016; Simon et al., 2018). Sexual cycles in the feline intestine result in the shedding of three to 810 million oocysts in a cat's faeces, three to five days after primary infection with *T. gondii* (Dabritz & Conrad, 2010), lasting for a median of 8 days (Dabritz & Conrad, 2010).

Cats seroconvert soon after they have shed oocysts, developing a long-lasting humoral immune response against *T. gondii* which is detectable using serological tests (Dubey & Frenkel, 1972; Dubey & Thulliez, 1989; Davis & Dubey, 1995; Dubey et al., 1995). Immunoglobin M (IgM) antibodies rise within days after primary infection and usually decrease over a few weeks. Immunoglobin G (IgG) antibodies rise after three to four weeks to protective levels; specific *T. gondii* IgG antibodies remain detectable for years and therefore seroprevalence is found to increase with age (Dubey & Thulliez, 1989; Dubey, 1995; Afonso et al., 2006; Lappin, 2010). Seroprevalence estimates in cat populations provide an

important indication of the extent of environmental contamination with *T. gondii* oocysts, an important first step in understanding disease prevalence and infection risk for intermediate hosts (Jones & Dubey, 2010; Fancourt & Jackson, 2014).

Several serological tests can be used to detect anti-*T. gondii* antibodies in cats, including agglutination tests, western blot, immunofluorescence, and enzyme-linked immunosorbent assays (ELISA) (Dubey & Thulliez, 1989; Lappin & Powell, 1991; Sohn & Nam, 1999; Dabritz et al., 2007; Györke et al., 2011; Opsteegh et al., 2012; Wyrosdick & Schaefer, 2015; Must et al., 2017) but evaluation of test characteristics – diagnostic sensitivity (Se) and specificity (Sp) – is a necessary prerequisite for interpretation of test results in serosurveys (Joseph et al., 1995; Greiner & Gardner, 2000; Mainar-Jaime & Barberán, 2007; Pan-ngum et al., 2013; Johnson et al., 2019). Test evaluation ideally should be carried out in the species and population in which the test is to be implemented (Greiner & Gardner, 2000; Mainar-Jaime & Barberán, 2007). This can be done by comparing test results to those obtained using a gold standard assay, which is assumed to be perfectly accurate (100 % Se and 100 % Sp), or using modelling approaches, such as Bayesian latent class analysis, which do not require the assumption that any test is perfect (Greiner & Gardner, 2000; Mainar-Jaime & Barberán, 2007).

Serological surveys conducted worldwide have shown that *T. gondii* infection is ubiquitous in wild and domestic cat populations (Dubey & Jones, 2008; Dabritz & Conrad, 2010; Lappin, 2010; Jokelainen et al., 2012). Global *T. gondii* seroprevalence is reported to be 30 – 40% for domestic cats (Elmore et al., 2010; Dubey, 2016) but estimates vary widely between and within populations, regions, and countries. Factors that affect seroprevalence are not fully understood but are known to include the age, sex, breed, and lifestyle of the cat (Dubey, 1973; Vollaire et al., 2005; Must et al., 2017), particularly whether the cat has outdoor access and engages in hunting behaviour (Opsteegh et al., 2012; Must et al., 2015). Estimates also depend on the serological test used and whether test accuracy is considered in seroprevalence estimation (Györke et al., 2011).

In New Zealand, toxoplasmosis is considered to be a problem for livestock and wildlife. Exposure to *T. gondii* is associated with suboptimal reproductive performance in sheep and farmed deer (West, 2002; Patel et al., 2017). Infection with *T. gondii* also appears to be prevalent in humans in New Zealand, with a seroprevalence of 43 % found amongst blood donors (Zarkovic et al., 2007), and may be an under-reported problem in pregnant women (Moor et al., 2000). In addition, fatal and latent cases of toxoplasmosis have been reported in certain species of endemic bird (Howe et al., 2014) and marine mammal (Roe et al., 2013, 2017). The domestic cat remains the only felid species found in New Zealand since its introduction in the mid-19th century (King & Barrett, 2005) and is therefore the only source of *T. gondii* oocysts in the country.

Cats are a favourite companion animal of New Zealanders, with approximately 44 % of all households owning 1.5 cats on average (Zito et al., 2019). New Zealand is also home to large populations of stray and feral domestic cats (Farnworth et al., 2013; Zito et al., 2019). It is thought that most cats in New Zealand have antibodies to *T. gondii* (Thompson, 1993, 1999), which indicates exposure to the parasite at some time during their lives and previous oocyst shedding, but evidence appears to be anecdotal. The present study therefore aimed to provide a preliminary estimate of true seroprevalence in naturally exposed companion cats in New Zealand.

3.2 Methods

3.2.1 Study rationale and overview

At the time of the study, there was no commercial cat-specific test available in New Zealand so results from three serological assays were compared. Two were commercial tests, the Toxotest-MT Eiken latex agglutination test (LAT) (Eiken Chemical Co., Ltd., Japan) and IDEXX Chekit-Toxotest IgG enzyme-linked immunosorbent assay (ELISA) for small ruminants (IDEXX laboratories, Switzerland). The third was an in-house anti-*Toxoplasma* IgG Western Blot (WB) (Patel et al., 2017), using an anti-cat secondary antibody. Diagnostic tests were evaluated with and without the WB as a gold standard (Sohn & Nam,

1999; Patel et al., 2017) using frequentist and Bayesian approaches, respectively. Demographic variables, including age, sex, and geographic location, were also available for the study cats, and so were investigated as potential risk factors for *T. gondii* seropositivity.

3.2.2 Samples

Two hundred serum samples were opportunistically selected from companion domestic cat samples submitted by veterinarians in 2012 to a commercial diagnostic laboratory (New Zealand Veterinary Pathology (NZVP) Ltd) for pre-surgery blood biochemistry testing or health screen blood work. Cats included in the study were apparently healthy. For the purpose of this study, companion (owned) cats are defined as cats that are considered to be owned by a specific person, sociable, and are directly dependent on humans (New Zealand National Cat Management Strategy Group, 2017) (for definitions of feral and stray domestic cats see Chapter 4). After completion of clinical testing, 200 µl of serum from each sample was aliquoted into a clean 1 ml microtube and stored at -20 °C until used for serological testing. As this diagnostic laboratory services the lower North Island and some regions of the South Island, samples included 166 cats from the North Island and 34 from the South Island. Samples were from eight distinct regions: Hawke's Bay (*n* = 5), Manawatu/Wanganui (*n* = 33), Taranaki (*n* = 6), Wellington (*n* = 122), Tasman (*n* = 15), Canterbury (*n* = 5), Otago (*n* = 12), and Southland (*n* = 2). Cat age and sex was also recorded if available.

3.2.3 Serological assays

All sera were screened for anti-*Toxoplasma gondii* antibodies using a commercial Latex Agglutination Test (LAT) (Toxoreagent, Eiken Chemical & Co, Tokyo, Japan). Sera were tested using 2-fold dilutions from 1:16 until the last reactive dilution. The LAT was performed according to the instructions of the manufacturer, whereby sera with endpoint titres < 1:32 were considered sero-negative, titres of 1:32 were considered weak sero-positive, and titres ≥ 1:64 were called positive. For analyses, endpoint titres of ≤ 1:32 were considered sero-negative, and titres ≥ 1:64 were initially considered sero-positive.

Assay performance was re-evaluated at optimised cut-offs following ROC curve analysis with the WB as gold standard.

Sera were then tested with a commercial anti-ruminant immunoglobin-G (IgG) based *T. gondii* ELISA test (Chekit-Toxotest, IDEXX laboratories, Bern, Switzerland), according to manufacturer's instructions. The status of a sample was evaluated by calculating the sample to positive (S/P) ratio (%). For the purpose of analysis, initially, sera with S/P (%) < 30 were considered seronegative, whereas sera with S/P (%) ≥ 30 were initially considered seropositive. Assay performance was re-evaluated at optimised cut-offs following ROC curve analysis with and without the WB as a gold standard.

Sera were also tested using an in-house anti-*T. gondii* IgG Western Blot (WB). The WB assay was performed as described in detail by Patel et al. (2017), except that the secondary antibody used was goat anti-cat IgG antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, Maryland, USA). Negative and positive (known cat positive) control sera were included in each WB run. Presence of bands indicating immunodominant antigens (IDA) of *T. gondii* from the positive control serum were recorded and study sera that recognised two or more IDA with molecular weight (MW) between 24 to 40 kD were considered positive for *T. gondii*. The positive control serum used in the study showed consistent bands at 24kD, 31kD, 33kD, 38kD, and 40kD and therefore acted as an internal control for loading and antibody probing.

3.2.4 Statistical Analysis

3.2.4.1 Western Blot as gold standard test

Results from the two commercial tests (LAT and ELISA) were first analysed using the in-house IgG WB as a gold standard (assuming 100 % Se and 100 % Sp) (Sohn & Nam, 1999; Patel et al., 2017) to estimate apparent seroprevalence, Se, and Sp. Receiver operating characteristic (ROC) curve analysis was also used to find optimal test cut-offs, assuming an equal cost of false positive and false negative, and to

compare diagnostic performance of the LAT and ELISA (Metz, 1978; Zweig & Campbell, 1993; Choi et al., 2006; Müller et al., 2009). The area under each ROC curve (AUC) was also calculated to provide an overall measure of test accuracy. Inter-assay agreement was further assessed by calculating the Kappa statistic (K) for the WB and LAT, and the WB and ELISA, where test agreement was interpreted as $K \leq 0.2$ = slight agreement, $0.2 < K \leq 0.4$ = fair, $0.4 < K \leq 0.6$ = moderate, $0.6 < K \leq 0.8$ = substantial, $K > 0.8$ = almost perfect agreement (Dohoo et al., 2013). Analyses were performed in R version 3.4.4 (R Core Team, 2017) using the package epiR version 0.9-96 (Stevenson et al., 2018) and in MedCalc for Windows, version 18.2.1 (MedCalc Software, Ostend, Belgium).

3.2.4.2 Bayesian latent class analysis assuming absence of a gold standard

Bayesian modelling was then used to simultaneously determine the posterior mean and 95 % credible interval (CrI) for true prevalence, sensitivity, and specificity of the three tests, assuming that the WB was not a gold standard (N = 138). The LAT and WB were considered to have a dichotomous outcome, whereas the ELISA outcome was considered to be continuous. A Bayesian ROC curve was also obtained for the ELISA to determine the optimal cut-off value. ELISA S/P (%) values were transformed using ln(S/P % + 1) to achieve approximate normality (Opsteegh et al., 2010; Giles et al., 2018) and were back-transformed for reporting of results. All sera were considered to come from one population. The three serological assays under evaluation were assumed to be conditionally dependent, given true disease status, because they have the same biological basis, i.e. the detection of anti-*T. gondii* IgG antibodies (Dendukuri & Joseph, 2001; Georgiadis et al., 2003; Opsteegh et al., 2010; Györke et al., 2011; Johnson et al., 2019). Bayesian analysis was performed using latent class models. True disease status of an individual cat (z_i) was modelled using a Bernoulli distribution with the probability of 'success' = true *T. gondii* seroprevalence. True disease status (z_i) was then incorporated into Bernoulli models for both WB and LAT outcomes, and Normal models for log-transformed ELISA outcomes. Conditional dependencies were introduced in a step-wise manner. Starting with the WB model, conditional dependency of the LAT on WB was achieved by having separate Se_{LAT} and Sp_{LAT} for each WB outcome (WB-pos or WB-neg). The log-transformed ELISA result was modelled using eight normal distributions, with a separate mean μ and precision τ (reciprocal of the variance = $1/\sigma^2$), depending on

true disease status (z_i), WB test outcome, and LAT test outcome. The full model assuming conditional dependence is over-parameterised, and therefore required prior information to be elicited for seroprevalence and test characteristics (Se and Sp). Beta prior distributions were used for seroprevalence (π), Se_{WB}, Sp_{WB}, $Se_{LAT\ (WB = 0)}$, $Se_{LAT\ (WB = 1)}$, $Sp_{LAT\ (WB = 0)}$, $Sp_{LAT\ (WB = 1)}$. Uniform priors were used for the mean (μ) and standard deviation (σ) of the eight log-transformed ELISA distributions. Prior information on *T. gondii* seroprevalence (Thompson 1993, 1999; Elmore et al. 2010; Dubey 2016), Se and Sp for the WB (Basso et al., 2013; Patel et al., 2017), LAT (Dubey et al., 1995; Kimbita et al., 2001; Ramos Silva et al., 2001; Patel et al., 2017), and ELISA (Mainar-Jaime and Barberán 2007; Opsteegh et al. 2010; Patel et al. 2017) was derived from published reports on cats and other species, and from expert opinion [L. Howe] (summarised in Table 4). For example, *T. gondii* seroprevalence was previously considered to be 100 % by LAT for domestic cats in New Zealand in 1992 (Thompson et al., 1993). However, this is likely to be an over-estimate because most cats examined were adults, and most domestic cats become infected with and develop immunity to *T. gondii* at an early age. Presenting signs which initiated a request for *Toxoplasma* examination in these cats were not provided for many of the cases (Thompson et al., 1993, 1999). Considering that the worldwide estimate of *T. gondii* seroprevalence in domestic cats is 30 – 40 % (Elmore et al., 2010; Dubey, 2016), the most likely seroprevalence estimate in this study population (π) was assumed to be 40 %, with 95 % confidence that the estimate was greater than 25 %, corresponding to a beta distribution with $\alpha = 9.68$, $\beta = 14.02$. Uniform priors were specified for the means of the log-transformed ELISA S/P (%) values for the non-diseased (μD-) and diseased cats (μD+) based on the range of log-transformed ELISA scores, and the expectation of a higher mean for D+ than D-, with μD- ~ uniform(-1, 3); μD+ ~ uniform(μD-, 5). Priors for μD- and μD+ were the same for each combination of the results of the WB and LAT. A uniform prior on the interval [0, 2] was specified for both σD- and σD+. Models were fit in JAGS version 4.3.0 (Plummer, 2003)) and R version 3.4.4 (R Core Team, 2017) using packages epiR version 0.9-96 (Stevenson, 2018), rjags version 4-6 (Plummer, 2016), and utility programs from DBDA2E-utilities.R (Kruschke, 2015)). The model was run with a burn-in of 1,000 iterations, and estimates were based on a further 5,000 iterations and 3 chains. Model convergence and chain stability was checked visually from the history plots and by comparing the posterior distributions from the three chains. The JAGS code for this model with conditional dependence between two dichotomous and one continuous test

for a single population is shown in Appendix 3i. The influence of the elicited priors on posterior estimates was evaluated through sensitivity analyses in which the prior distributions were slightly to somewhat perturbed (see Table 4), as suggested by Johnson et al. (2019). The use of Bayesian models for diagnostic test evaluation is reviewed in detail elsewhere (e.g. Dendukuri & Joseph, 2001; Johnson et al., 2001; Georgiadis et al., 2003; Branscum et al., 2005; Jones et al., 2010; Wesley et al., 2019).

3.2.5 Risk factor analysis

To evaluate demographic variables as risk factors for *T. gondii* seropositivity, binomial generalised linear models (GLM) with logit link functions were fitted to the data, with ELISA result (positive if S/P ≥ 30, otherwise negative) as the outcome variable. Univariate analyses were first performed for cat age (in years), sex, and island (North/South), to estimate crude odds ratios and 95 % confidence intervals (CI). Demographic variables associated with ELISA results ($p \leq 0.20$) were incorporated into a multivariable model (Opsteegh et al., 2012; VanWormer et al., 2013). Using the multivariate model, adjusted odds ratios with 95 % CI were estimated to assess the strength of the association between each risk factor and ELISA result. Cats with missing data were omitted from analyses. Data visualisation and statistical analyses were performed in R version 3.4.4 (R Core Development Team 2017), using ggplot2 (Wickham 2016), dplyr (Wickham et al., 2018), binom v 1.1-1 (Dorai-Raj, 2014), lme4 v 1.1-17 (Bates et al., 2015), generalhoslem (Jay, 2018), tidyverse (Wickham, 2017), broom (Robinson & Hayes, 2018), car (Fox & Weisberg, 2011).

3.3 Results

3.3.1 Western Blot

Of the 200 serum samples obtained, 138 had sufficient volume (> 20 µl) and quality and were tested using the in-house anti-*Toxoplasma* IgG WB, resulting in a seroprevalence estimate of 43 % (59/138; 95 % CI: 35 - 51).

3.3.2 Latex Agglutination Test

Using the endpoint titre of ≥ 1:64 as the cut-off, 75.5 % (151/200) of the serum samples were found to be sero-positive for anti-*T. gondii* antibodies. Using the WB as a gold standard test (*n* = 138), and ≥ 1:64 as the cut-off to distinguish between positive and negative samples, apparent seroprevalence was estimated to be 78 % (107/138; 95 % CI 70 – 84). Se_{LAT} was determined to be 95 % (95 % CI: 86 – 99) and Sp_{LAT} to be 35 % (95 % CI: 25 – 47). The Kappa co-efficient was estimated to be 0.28 (fair agreement). ROC analysis using 1:16, 1:32, 1:64, 1:128 as cut-offs for the LAT revealed that the optimal cut-off was an end-point titre ≥ 1:128, giving a Se_{LAT} of 90 % (95 % CI: 79 – 96) and Sp_{LAT} of 54 % (95 % CI: 43 – 66), and an apparent prevalence of 64 % (95 % CI: 56 – 72) (see Figure 5 and Table 5). The AUC was calculated to be 0.73 (95 % CI: 0.648 – 0.802). At the optimised cut-off the Kappa value increased to 0.42 (moderate agreement).

3.3.3 ELISA

Overall, 67 % (134/200) of sera were positive with an S/P ratio of ≥ 30 %, with 42 % (83/200) strongly positive with an S/P ratio of ≥ 100%. Using the WB as a gold standard test, and S/P ≥ 30% as the cut-off, Se_{ELISA} was determined to be 95 % (95% CI: 86 – 99), Sp_{ELISA} to be 63 % (95 % CI: 52 – 74), and apparent seroprevalence was estimated to be 62 % (85/138; 95 % CI 53 – 70). The Kappa co-efficient was estimated to be 0.55 (moderate agreement). ROC analysis revealed that the optimal cut-off for the ELISA was an S/P ratio > 62%, giving a Se_{LAT} of 85 % (95 % CI: 73 – 93) and Sp_{LAT} of 80 % (95 % CI: 69 – 88), and an apparent prevalence of 48 % (95 % CI: 39 – 56) (see Figure 6 and Table 5). The AUC was calculated to be 0.872 (95 % CI: 0.805 – 0.923). Comparison of the ROC curves for the ELISA and LAT showed that there was a statistically significant difference between the areas under the two curves ($P < 0.0001$), meaning that, compared with the LAT, the ELISA is a significantly more accurate test using optimised cut-offs and assuming the WB to be a gold standard. This was reflected in the updated Kappa co-efficient, which was estimated to be 0.64 (substantial agreement).

3.3.4 Bayesian analysis in the absence of a gold standard

A Bayesian modelling approach was then applied to the data (*N* = 138) to estimate *T. gondii* seroprevalence and the accuracy of each diagnostic test, without assuming that the WB was a perfect gold standard. The model included all three diagnostic tests, the WB, LAT, and ELISA, with conditional dependence. Results are summarised in Table 6. Using this model, the prevalence of *T. gondii* infection in the study population was estimated to be 61 % (95 % CrI 48 – 76). The Se and Sp of the WB were determined to be 66 % (95 % CrI 52 – 81) and 95 % (95 % CrI 86 – 99), respectively, whereas the Se and Sp for the LAT were estimated to be 87 % (95 % CrI 78 – 93) and 55 % (95 % CrI 36 – 81) (posterior distribution plots are available in Appendix 3ii. Using ROC curve analysis, the cut-off which maximised both Se and Sp for the ELISA was found to be in accordance with the manufacturer's recommendation, i.e. a S/P % ≥ 30, giving Se_{ELISA} of 84 % (95 % CrI 73 – 93) and Sp_{ELISA} of 86 % (95 % CrI 77 – 97) (see Figure 7). Sensitivity analysis was carried out by perturbing the priors by small to modest amounts (Johnson et al., 2019). Parameter estimates from the sensitivity analyses were similar to those of the original analysis ($-5 \leq x \leq 8$ %) and were within the original credible intervals (results of the sensitivity analysis are shown available in Appendix 3iii.

3.3.5 Risk factor analysis

Of the 200 cats included in the study, *n* = 191 had sex recorded. Of these, 92 were male (48 %) and 99 were female (52 %). Age data was available for *n* = 190 cats. Age ranged from 0.5 to 19 years, with a median of 12 years, lower quartile of 10 years, and upper quartile of 14 years. All 200 cats in the study had been tested for *T. gondii* antibodies using the ELISA and 67 % were positive with a cutoff of S/P ≥ 30 (see above). Both age and sex were significantly associated with ELISA results in univariate analyses ($p = 0.00525$ and $p = 0.035$, respectively). Seventy-five percent (69/92) of males were positive (95 % CI: 65.3 – 82.7) compared with 60/99 (60.6 %) females (95% CI: 50.8 – 69.7). The distributions of ages for cats testing positive and negative are shown in Figure 8. Multivariate analysis showed that the odds of testing positive on the ELISA were nearly two and half times as high for male cats than female cats (OR = 2.49, 95 % CI: 1.30 – 4.95, $p = 0.0072$), and that the odds of getting a positive result increased by 15 %

with each additional year of age (OR = 1.15, 95 % CI: 1.06 – 1.25, p = 0.0014). ELISA results were not significantly associated with island (p = 0.376). Sample sizes from several regions were too small for the study to have sufficient power to assess regional risk of seropositivity.

Table 4 Expert-elicited values and prior distributions used for estimation of true *Toxoplasma gondii* seroprevalence in New Zealand companion cats, sensitivities (Se) and specificities (Sp) of a western blot (WB), latex agglutination test (LAT), and enzyme-linked immunosorbent assay (ELISA) in a Bayesian latent class model, with dependence between the three tests and one population

Parameter	Parameter Description	Expert Opinion	Parameter Prior Distribution	Alternative Prior for Sensitivity Analysis
π	Seroprevalence	Mode = 0.4, and 95% sure that mode > 0.25	Be(9.6816, 14.0224)	Be(7.4783, 9.5875)
SeWB	Se WB	Mode = 0.71, and 95% sure that mode > 0.5	Be(12.3804, 5.6483)	Be(10.8353, 4.6377)
SpWB	Sp WB	Mode = 0.85, and 95% sure that mode > 0.5	Be(6.252, 1.9268)	Be(6.9618, 2.3087)
SeLATc[1]	Se LAT when WB negative	Mode = 0.72, and 95% sure that mode > 0.5	Be(11.5581, 5.1059)	Be(10.1963, 4.2311)
SeLATc[2]	Se LAT when WB positive	Mode = 0.72, and 95% sure that mode > 0.5	Be(11.5581, 5.1059)	Be(10.1963, 4.2311)
SpLATc[1]	Sp LAT when WB negative	Mode = 0.86, and 95% sure that mode > 0.5	Be(6.0514, 1.8223)	Be(6.7051, 2.1685)
SpLATc[2]	Sp LAT when WB positive	Mode = 0.86, and 95% sure that mode > 0.5	Be(6.0514, 1.8223)	Be(6.7051, 2.1685)
μD-	log-transformed ELISA mean for uninfected cats		U[-1, 3]	
μD+	log-transformed ELISA mean for infected cats		U[μD-, 5]	
σD-	log-transformed ELISA standard deviation for uninfected cats		U[0, 2]	
σD+	log-transformed ELISA standard deviation for infected cats		U[0, 2]	

Table 5 Apparent seroprevalence and test characteristics (sensitivity (Se), specificity (Sp), and Kappa statistic) with 95 % confidence intervals for the latex agglutination test (LAT) and enzyme-linked immunosorbent assay (ELISA). Both the manufacturer's recommended cut-off and an optimised cut-off which maximised both Se and Sp (determined using receiver operating characteristic (ROC) curve analysis) were used to distinguish between infected and uninfected cats. All values were calculated assuming that the western blot (WB) was a gold standard reference test

Test	**LAT**		**ELISA**	
Cut-off	**titre ≥ 1:64[1]**	**titre ≥ 1:128$_{ROC}$[2]**	**S/P % ≥ 1:30[1]**	**S/P % > 1:62$_{ROC}$[2]**
Parameter				
Apparent seroprevalence (%)	78 (70 – 84)	64 (56 – 72)	62 (53 – 70)	48 (39 – 56)
Sensitivity (%)	95 (86 – 99)	90 (79 – 96)	95 (86 – 99)	85 (73 – 93)
Specificity (%)	35 (25 – 47)	54 (43 – 66)	63 (52 – 74)	80 (69 – 88)
Kappa (K)[3]	0.28 (0.12 – 0.43)	0.42 (0.27 – 0.56)	0.55 (0.42 – 0.69)	0.64 (0.51 – 0.76)

[1] manufacturer's recommended cut-off

[2] optimised cut-off as determined by receiver operating characteristic (ROC) curve analysis

[3] where $K \leq 0.2$ = slight agreement, $0.2 < K \leq 0.4$ = fair, $0.4 < K \leq 0.6$ = moderate, $0.6 < K \leq 0.8$ = substantial, $K > 0.8$ = almost perfect agreement (Dohoo et al., 2013)

Table 6 Test sensitivity (Se) and specificity (Sp) with 95 % credible intervals for the western blot (WB), latex agglutination test (LAT), and enzyme-linked immunosorbent assay (ELISA), as determined by Bayesian latent class analysis, using the manufacturer's recommended cut-off and an optimised cut-off for the ELISA which maximised both Se and Sp, determined using receiver operating characteristic (ROC) curve analysis. Parameter estimates are given by the mean of each posterior distribution

Test	**WB**	**LAT**	**ELISA**
Cut-off		**titre ≥ 1:64[1]**	**S/P % ≥ 1:30[2]**
Parameter			
Sensitivity (%)	66 (52 – 81)	87 (78 – 93)	84 (73 – 93)
Specificity (%)	95 (86 - 99)	55 (36 – 81)	86 (77 – 97)

[1] manufacturer's recommended cut-off

[2] manufacturer's recommended cut-off and optimal cut-off as determined by receiver operating characteristic (ROC) curve analysis

Figure 5 Receiver Operating Characteristic (ROC) curve plotted for the Latex Agglutination Test (LAT) from analysis with the Western Blot (WB) as a gold standard, where cut-off criteria were categorised as follows: 0 if the end-point titre was 0; 1 if the end-point titre was 1:16, 2 if the titre was 1:32, 3 if the titre was 1:64, and; 4 if the titre was 1:128 or higher. The optimal cut-off criterion which maximised both sensitivity (Se) and specificity (Sp) and associated test characteristics are shown in the legend. The thick blue line represents the true positive rate (Se) plotted as a function of the false positive rate (100 - Sp) for different test cut-off points. The dotted blue lines represent the 95 % confidence bounds for the ROC curve values. The red diagonal is the line of no discrimination, which represents a test that randomly classifies results as positive or negative.

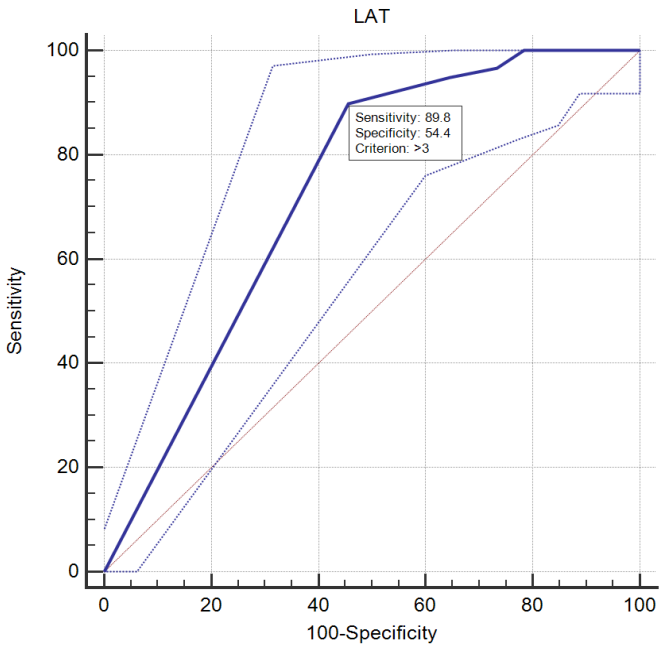


Figure 6 Receiver Operating Characteristic (ROC) curve plotted for the ELISA from analysis with the Western Blot (WB) as a gold standard. The optimal cut-off criterion (S/P %) which maximised both sensitivity (Se) and specificity (Sp) and associated test characteristics are shown in the legend. The thick blue line represents the true positive rate (Se) plotted as a function of the false positive rate (100 - Sp) for different test cut-off points. The dotted blue lines represent the 95 % confidence bounds for the ROC curve values. The red diagonal is the line of no discrimination, which represents a test that randomly classifies results as positive or negative.

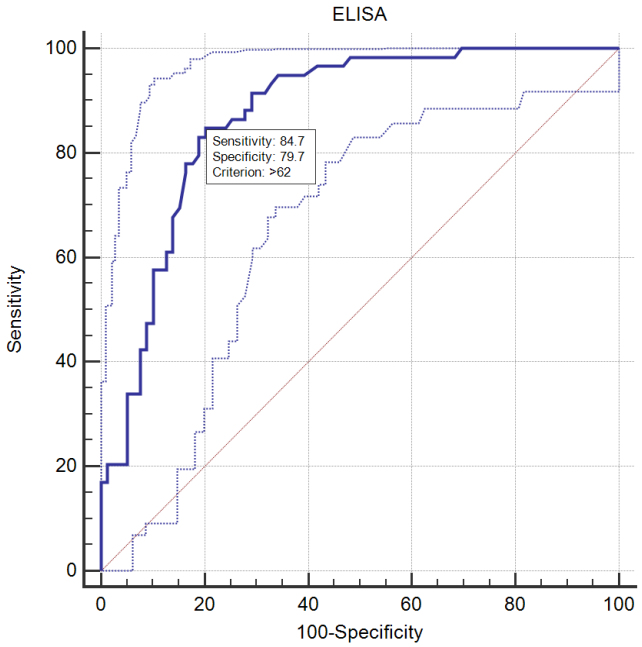


Figure 7 Receiver Operating Characteristic (ROC) curve plotted for the log-transformed ELISA results following Bayesian latent class analysis. The optimal cut-off criterion, which maximised both sensitivity (Se) and specificity (Sp), and associated test characteristics are given in the legend. The ROC curve plots the true positive rate (Se) against the false positive rate (100 - Sp). The blue point is where Se is maximised and 100 - Sp is minimised. The optimal cut-off was found to be equivalent to the manufacturer's recommended cut-off of S/P % ≥ 30

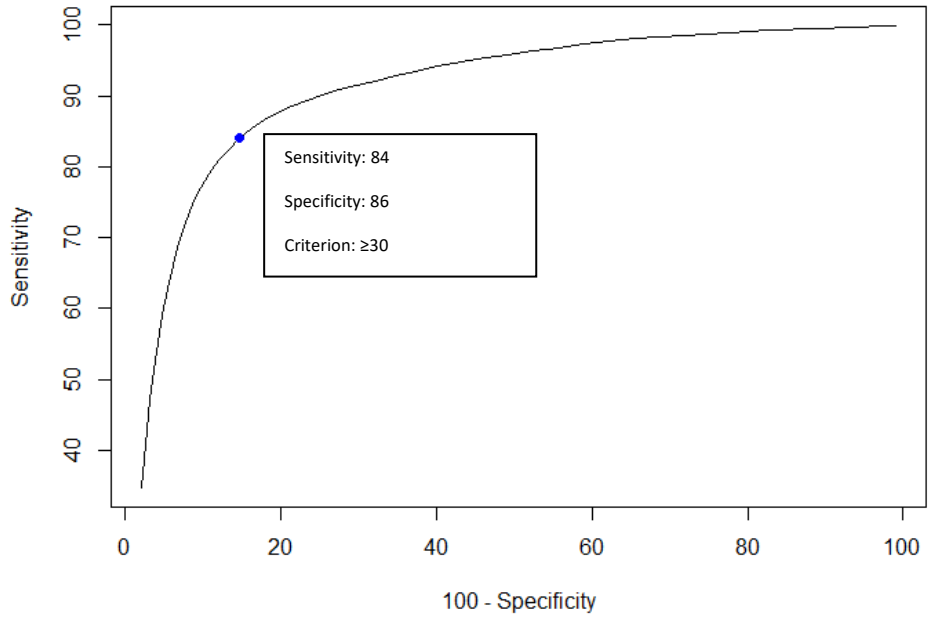
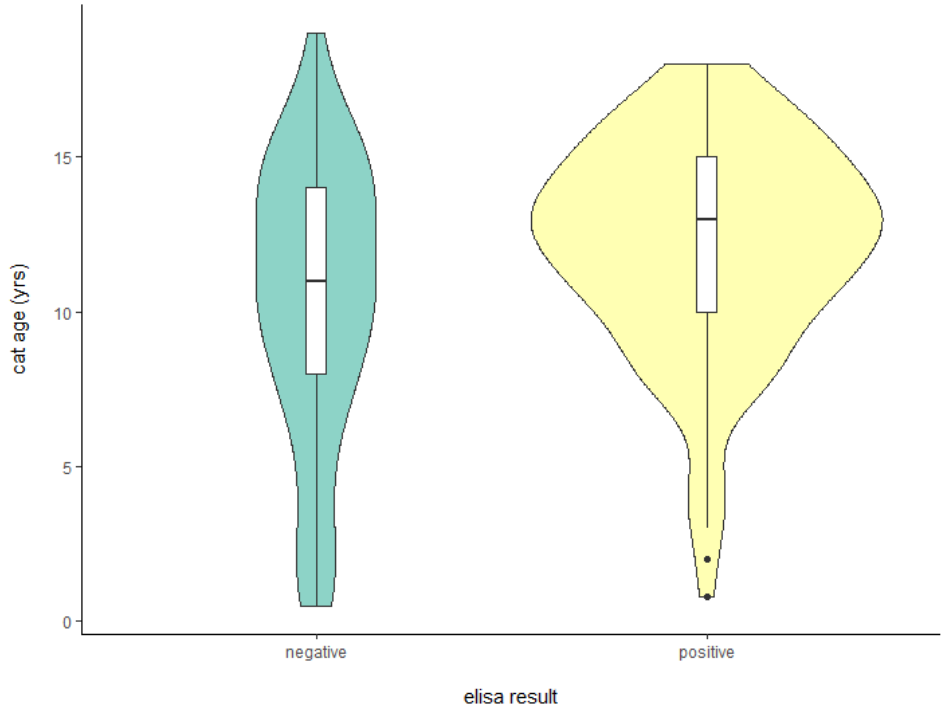


Figure 8 This violin plot shows the relationship between seropositivity for *T. gondii* and cat age. The box plot elements show the median age of the study cats, the interquartile age range, and the rest of the age distribution. The median age is shown to be lower for seronegative cats (median = 11 yrs, IQR = 8 – 14 yrs) compared with seropositive cats (median = 13 yrs, IQR = 10 – 15 yrs). On each side of the boxplots, kernel density estimates show the distribution shape of the data. Wider sections of the violin plot represent a higher probability that cats in the study population will take on the given value; thinner sections represent a lower probability. The shape of the distribution indicates that cat ages are highly the ages of seropositive cats are highly concentrated about the median. Plots are scaled by the number of cats in each group (seronegative, *n* = 63; seropositive, *n* = 127).



3.4 Discussion

This study is the first to screen serum samples from companion cats in New Zealand to investigate *T. gondii* infection in the domestic cat population, as indicated by seropositivity. Using Bayesian latent class analysis, true seroprevalence was determined to be 61 % (95 % CrI: 48 – 76), showing that *T. gondii* exposure and thus oocyst shedding (Jones & Dubey, 2010) is widespread in companion cats in New Zealand. This finding indicates that there may be a high risk of infection for a range of

intermediate hosts in the country (Fancourt & Jackson, 2014), supported by high seroprevalence estimates in non-feline species previously surveyed in New Zealand. For example, seroprevalence was estimated to be 61 % for non-vaccinated ewes (Dempster et al., 2011) and 33 % in farmed red deer (Patel et al., 2017) and infection with *T. gondii* also appears to be prevalent in humans in New Zealand, with a seroprevalence of 43 % found amongst blood donors (Zarkovic et al., 2007). The Bayesian analysis carried out in this study was novel, including two binary (LAT and WB) and one continuous (ELISA) test administered on individual cats from one population, with conditional dependence between all three tests. Determining whether the model was identifiable was beyond the scope of this study, but it should be noted that the posterior estimates may be reliant on the validity of prior information used, although in the small sensitivity analysis conducted, they were not found to change substantially from the original results.

The New Zealand seroprevalence estimate provided by this study is high relative to the global *T. gondii* seroprevalence estimate in domestic cats of 30 – 40 % (Elmore et al., 2010). Looking to New Zealand's nearest neighbours, the New Zealand estimate of 61% is higher than seroprevalence in companion cats in Melbourne, Australia (39 %; Sumner & Ackland, 1999), and higher than a recent nationwide Australian estimate of 38 % (*n* = 425), also in owned cats (Brennan, 2015), although authors of these studies did not consider test characteristics when calculating seroprevalence. In general, it is difficult to compare results across surveys of domestic cats because of differences in serological tests used, and because most feline serosurveys do not provide test characteristics needed to calculate true prevalence (Jones & Dubey, 2010). It is also the case that estimates actually vary widely both within and between countries, regions, and populations of cats tested; overall study seroprevalence estimates range between 5.6 and 97 % worldwide (Al-kappany et al., 2010; Dabritz & Conrad, 2010; Jones & Dubey, 2010; Fancourt & Jackson, 2014) but seroprevalence can vary between 0 and 100 % across individual locations within the study area (Fancourt & Jackson, 2014; Rengifo-Herrera et al., 2017). Prevalence is also known to be higher in feral and stray than companion (owned) domestic cats in any given region (Dubey et al., 2006; Castillo-Morales et al., 2012; Cong et al., 2016), and also depends on the age

structure of the cat population. Nevertheless, results clearly demonstrate that *T. gondii* infection is prevalent in companion cats throughout New Zealand.

Seroprevalence results in this study are likely be affected by the age distribution of cats sampled. In cats, *T. gondii* infections are usually asymptomatic but tissue cysts and anti-*T. gondii* antibodies can persist for years after primary infection, possibly for the lifetime of the animal (Dubey & Thulliez, 1989; Dubey, 1995; Afonso et al., 2006; Lappin, 2010). Primary infection is thought to occur shortly after a cat is first allowed outside, usually after a kitten is weaned and begins to hunt (Dubey & Thulliez, 1989; Dubey, 1995). Seropositivity therefore tends to be associated with cat age within a population (Afonso et al., 2006, 2010). In the present study, the median age of the cats was 12 years and very few kittens (aged 1 year or younger) were sampled; the cats included in this study may be older on average than those in studies with lower *T. gondii* seroprevalence estimates. For example, seroprevalence in companion cats in the Netherlands was estimated to be 18.2 % (95 % CI: 16.6 – 20) by Opsteegh et al. (2012), and in that study many young cats were sampled, particularly cats aged 0.25 – 1.5 years (mean age being 6.5 years). As many older cats were included in the present study, there may have been an upward bias on the seroprevalence estimate. The age distribution of the New Zealand companion cat population is not known but if there was an overrepresentation of older cats in this study, seroprevalence for the total New Zealand cat population is probably somewhat lower than the given estimate of 61 %, a limitation previously discussed by Opsteegh et al. (2012).

Nevertheless, widespread exposure of New Zealand companion cats in this study may also be attributed to their having a high level of outdoor access compared to owned cats in other countries (Farnworth et al., 2010; Hall et al., 2017). As such, they have ample opportunity to engage in hunting behaviour and an increased probability of ingesting infective tissue cysts in prey (Dabritz et al., 2007). A strong positive association between *T. gondii* infection in domestic cats and levels of predation or outdoor access (Tenter et al., 1994; Gauss et al., 2003; Dabritz et al., 2007; Lopes et al., 2008; Opsteegh et al., 2012) has been documented in several previous studies. For example, in a serosurvey of

domestic cats in Northeastern Portugal (*n* = 204), Lopes et al. (2008) found that seroprevalence in cats kept totally indoors (7.7 %) was significantly lower than in those that were given outside access (45.4 %). Similarly, Opsteegh et al. (2012) found hunting behaviour amongst Dutch domestic cats to be a highly significant predictor of *T. gondii* infection.

Worldwide, several different assays are in use for the detection of antibodies against *T. gondii* in cats and many feline serosurveys have now been published. At present, the preferred test is the modified agglutination test (MAT) (Dubey & Desmonts, 1987), or its commercially-available counterpart, the Toxo-Screen DA kit (BioMerieux, France) (Tenter et al., 1994) as it is reportedly one of the most sensitive and specific tests available (Dubey & Thulliez, 1989; Tenter et al., 1994; Dubey et al., 1995; Dubey, 1997; Macrì et al., 2009; Györke et al., 2011). A number of cat-specific or multi-species, in-house or commercial, ELISAs have also been developed and reportedly perform best, alongside the MAT, for detecting IgG antibodies in cats (e.g. Dabritz et al., 2007; Györke et al., 2011; Opsteegh et al., 2012). As highlighted by Patel et al. (2017), these assays are unfortunately not available in New Zealand. Veterinary diagnostic laboratories in New Zealand use the LAT, and the only other assays available in the country at the time of this study were the commercial IgG ELISA (IDEXX Chekit Toxotest) for small ruminants, and in-house WB (Patel et al., 2017). Although the LAT has been used by a few authors as a confirmatory assay (Brown et al., 2005; Abdelbaset et al., 2017), studies have shown that it is not the most sensitive and specific assay for detecting anti-*T. gondii* antibodies in cat sera (Dubey & Thulliez, 1989; Silva et al., 2001). Whilst the LAT is known to be less sensitive and specific than other tests (Dubey & Thulliez, 1989; Silva et al., 2001), test characteristics for the ELISA and WB had not previously been evaluated in felids. For this reason, this study aimed to evaluate the performance of the three assays for use in New Zealand cats. Of particular interest was whether the ELISA is appropriate for use with cat sera as this test is relatively rapid and can be automated, reducing intra- and inter-user variation, and enabling the rapid screening of large numbers of samples (Georgiadis et al., 2003). In contrast, the in-house WB is laborious and time-consuming, and the LAT is subjective in interpretation, reliant on user observation of the agglutination endpoint – limitations discussed in Patel

et al. (2017). Although it is not always carried out, diagnostic test evaluation is also considered a prerequisite before any conclusions can be drawn from test results (Györke et al., 2011).

Bayesian latent class analysis showed that the best performing test in this study, taking into account both Se and Sp, was the ELISA. Using the manufacturer's recommended cut-off, determined to be optimal by Bayesian ROC curve analysis, ELISA performance was reasonable (Se = 84 %; Sp = 86 %). The Se of the LAT was comparable, estimated at 87 %, but Se of the WB was poor at 66%. In contrast, specificity of the WB was good, estimated to be 95 %, whilst it was 86 % for the ELISA and only 55% for the LAT. These tests have not previously been evaluated by means of Bayesian latent class analysis in cat populations. However, recently in New Zealand, Patel et al. (2017) found the same IDEXX Chekit Toxotest ELISA (manufacturer's cut-off) to have a Se of 85.1 % (76.2 – 91.9) and Sp of 98.5 % (96.9 – 99.4) for use in deer. Overseas, in sheep the test also performed well; through Bayesian analysis Opsteegh et al. (2010) found this ELISA to have a Se of 92.0 % (85.5 – 95.7) and Sp of 98.7 % (96.0 – 99.6), results comparable to an earlier study, also in sheep (Mainar-Jaime & Barbarán, 2007). Lower characteristics for the same test were seen in pigs, where Bayesian analysis resulted in a Se of 71.5 % and Sp of 85.5 % (Georgiadis et al., 2003). This disparity in test characteristics between sheep and pigs led Mainar-Jaime & Barbarán, 2007, to conclude that diagnostic accuracy of a test differs between animal species, an outcome supported by findings of this study, and others (e.g. Owen & Trees, 1998; Nielsen & Gall, 2001). This ELISA likely performs better in deer and sheep rather than pigs and cats due to the inclusion of a ruminant-specific conjugate in the kit, as although it is a multi-species test, pigs and cats do not belong to this suborder of mammals.

Despite this, the diagnostic accuracy of the LAT, preferred in New Zealand diagnostic laboratories because it does not rely on a species-specific conjugate, was found by Bayesian modelling to be inferior to that of the ELISA. The Se and Sp of the LAT for testing cat sera for specific *T. gondii* antibodies were previously estimated to be 72.5 % (58 – 84) and 86.5 % (71 – 95), using the MAT as a gold standard (Silva et al., 2001). In pigs, using bioassay as the gold standard, LAT test characteristics were estimated

as Se = 45.9 % and Sp = 96.9 % (Dubey et al., 1995). Recently in deer, Se and Sp of the LAT were estimated by Bayesian analysis to be 76.2 % (66.7 – 84.5) and 89.7 % (84.5 – 93.9), respectively (using a cut-off of ≥ 1:64 as employed by this study) (Patel et al., 2017). The sera in the deer study were also tested in New Zealand diagnostic laboratories, adding weight to the conclusion that a test's diagnostic accuracy is dependent on species under investigation. Results of the present study confirm that the LAT is not the most sensitive or specific assay in use today to detect *T. gondii* antibodies in cats, as well as other animals.

Bayesian modelling showed that the WB may not be suitable as a gold standard test for cats in New Zealand, despite the use of a cat-specific secondary antibody in this assay. While the WB was highly specific (95 %), sensitivity was relatively low (66 %). It is not clear why the sensitivity of the WB was low, but it could possibly be explained by general limitations of diagnostic test comparisons, which use the agreement between tests for estimation of test characteristics (Opsteegh et al., 2012). Although less likely using Bayesian analyses, as all tests are compared simultaneously, and prior information can be incorporated (Györke et al., 2011), if two tests misclassify the same sera in the same direction, this could lead to overestimation of test characteristics for these tests, and possible underestimation for a new test, assuming the new test is accurate for those sera. In the context of this study, if both the LAT and ELISA gave false positive results for the same sera, this could have led to sensitivity of the WB being underestimated. Results of this study cannot confirm the WB to be a gold standard for cats and suggest that the WB is not a sensitive screening assay. However, the high specificity of the WB points to this assay being useful for confirmatory purposes, for example, to rule in ELISA positives as true positives.

Performance characteristics of the LAT and ELISA were also evaluated considering the WB to be a gold standard reference test even though test characteristics were not known for its use in cats at the onset of the study. This was done and considered a useful preliminary analysis because the WB has previously been used as a gold standard to detect *T. gondii* antibodies in feline (Sohn & Nam, 1999), ovine (Wastling et al., 1994, 1995), and cervid (Patel et al., 2017) species, and the WB was the only cat-

specific test included in this study. This assay also performed very well and was considered suitable as a future gold standard test for use in deer in New Zealand, as Bayesian analysis showed the test to have Se and Sp approaching 100% (Se = 95.8 %; Sp = 95.1 %) (Patel, 2016; Patel et al., 2017). In the present study, gold standard analysis showed that the ELISA out-performed the LAT, even after cut-offs were optimised using ROC curves (SE_{LAT} was slightly higher than Se_{ELISA} when the cut-offs were optimised for both tests, but Sp_{LAT} remained poor in comparison). Therefore, despite expected differences between the gold standard and Bayesian results, overall conclusions were consistent.

Overall, the ELISA was found to be the best-performing assay in this study. Therefore, ELISA results were used to study risk factors for *T. gondii* infection in univariate and multivariate analyses by logistic regression (Györke et al., 2011). Commonly identified risk factors for infection in cats include increasing age, outdoor access, feeding raw meat and organs, and hunting. Factors less often identified include gender, geographic location, breed, contact with other cats and living in groups, and meteorological conditions (Afonso et al., 2007, 2010; Gauss et al., 2003; Lopes et al., 2008; Györke et al., 2011). Only gender, age, and sampling region were available for investigation here because the study was not set up to examine risk factors in depth. Both age and sex were significantly associated with being seropositive. The odds of being seropositive increased with age, by 15 % with each additional year (OR = 1.15, 95% CI: 1.06 – 1.25, $p = 0.0014$). This result was expected because with a persistent infection such as *T. gondii*, the prevalence of antibodies is known to increase with age (Tenter et al., 1994; Gauss et al., 2003; Miró et al., 2004; Afonso et al., 2006; Lopes et al., 2008; Opsteegh et al., 2012).

Controlling for age, male cats were found to have a greater odds of being seropositive than females, which was a somewhat unexpected result, as many overseas studies found no significant difference in seroprevalence between the sexes in domestic cats (D'Amore et al., 1997; Dorny et al., 2002; Smielewska-Loś & Pacoń, 2002; Lopes et al., 2008; Opsteegh et al., 2012). It is possible that the observed association between gender and seropositivity seen in this study could be a result of confounding, as other major known risk factors for infection, notably whether the cat hunted/had

outdoor access, or was fed raw meat (Must et al., 2017), were not reported or included in the models. However, a significantly higher seroprevalence in males than in females was also observed by Afonso et al. (2007), testing feral domestic cats from the Kerguelen Islands in the Indian Ocean. The difference in seropositivity between the genders in the Kerguelen Island cats was attributed to differences in body weight and diet composition. Whether this is the case in New Zealand remains to be determined. In the present study, island and region of sampling were not found to be associated with seropositivity, although the analysis for region was underpowered as sample sizes for some regions were very small. Results suggests that seroprevalence is consistent across all regions sampled, demonstrating a high level of *T. gondii* contamination throughout New Zealand. Results also suggest that *T. gondii* infected prey is prevalent across New Zealand, considering predation to be the main source of infection for cats (Davis & Dubey, 1995; Dubey, 2016; Simon et al., 2018).

The domestic cat is thought to be the most important host species of *T. gondii* evolutionarily and epidemiologically, particularly in regions where domestic cats outnumber other felids (Jokelainen et al., 2012; Must et al., 2017). Most seropositive cats are considered to have previously shed oocysts in their faeces (Jones & Dubey, 2010). Accordingly, the high *T. gondii* seroprevalence observed in this study suggests that a fairly large proportion of companion cats in New Zealand have shed *T. gondii* oocysts into the environment, indicating that there exists a high risk of infection for susceptible intermediate hosts. It is, however, important to interpret results taking into consideration the age distribution of the cats included in the study. As the median age was high, with few younger cats tested, it may not be appropriate to extrapolate results of this study to the entire companion cat population of New Zealand. It should also be considered that test Se and Sp values may be affected by the age structure of study population (Nielsen & Toft, 2006). Future research should aim to include a more representative sample of the New Zealand cat population in terms of age for seroprevalence estimation and, whilst the ELISA used in this study seems to be acceptable for determining *T. gondii* seroprevalence in New Zealand cats, introduction and validation of the MAT or its commercial counterpart in New Zealand would be ideal. Another limitation of the present chapter is that the serological tests used cannot distinguish between recent and long-term infections, meaning they cannot confirm precisely when the cat was

shedding oocysts. Future investigation of oocyst shedding in New Zealand should be conducted either through investigating the dynamics of seroconversion in cats (Afonso et al., 2009) or directly through faecal examination (Chapter 4).

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4 *T. gondii* in New Zealand cats: oocyst shedding study

4.1 Introduction

Environmental transmission of *Toxoplasma gondii* oocysts poses a health threat for warm-blooded terrestrial and aquatic animals, thus knowledge of feline infections and the environmental oocyst burden is essential (Jokelainen et al., 2012). Serological studies have shown that exposure to *T. gondii* is widespread in cat populations across the globe (Dubey & Jones, 2008; Dabritz & Conrad, 2010; Lappin, 2010; Jokelainen et al., 2012; Chapter 3) but direct detection of *T. gondii* in cat faeces is necessary to estimate the proportion of cats actively shedding oocysts (Dabritz & Conrad, 2010; Jokelainen et al., 2012). *Toxoplasma gondii* undergoes sexual reproduction in the feline intestine, resulting in the production of millions of oocysts which are shed in faeces three to five days after infection (Dabritz & Conrad, 2010). The shedding period lasts for a median of eight days, up to three weeks (Dubey, 1976, 2001, 2002, 2005). Oocyst shedding is mainly thought to occur only once in a cat's lifetime after initial infection, usually when a young cat is weaned and starts to hunt (Dubey & Frenkel, 1974; Fritz et al., 2012), but re-infection and re-shedding can occur (Dubey, 1976, 1995; Zulpo et al., 2018). Oocysts shed in cat faeces can accumulate and survive for months to years in soil, freshwater, and seawater, which allows for their transmission to intermediate hosts such as rodents, birds, sheep, humans, and even marine mammals (Yilmaz & Hopkins, 1972; Frenkel et al., 1975; Lélu et al., 2012; Howe et al., 2014; VanWormer et al., 2014).

Direct detection of *T. gondii* oocysts in cat faeces is usually carried out by faecal floatation, which may be coupled to polymerase chain reaction (PCR) for confirmatory purposes (Schares et al., 2008; Dabritz & Conrad, 2010). The reported prevalence of *T. gondii* oocysts in domestic cat faeces worldwide ranges from 0 – 34 % (summarised by Dabritz & Conrad, 2010; Dubey et al., 2013; Chemoh et al., 2016; Veronesi et al., 2017), but is typically <2 % where the estimate is based on flotation and light microscopy (e.g., Miró et al., 2004; Pena et al., 2006; Dabritz et al., 2007; Karatepe et al., 2008; Schares

et al., 2008, 2016; Herrmann et al., 2010; Mancianti et al., 2010; Berger-Schoch et al., 2011; Jokelainen et al., 2012). Direct detection of the parasite also enables genetic characterisation of *T. gondii* strains entering the environment (Schares et al., 2008; Jokelainen et al., 2012), which is particularly important for tracking the source of host infections (Miller et al., 2008; VanWormer et al., 2014).

Historically, *T. gondii* was thought to have a simple population structure comprised of three archetypal lineages, Type I (ToxoDB #10), Type II (ToxoDB #1 and #3), and Type III (ToxoDB #2), based on restriction fragment length polymorphism (RFLP) analyses (Howe & Sibley, 1995). Since then, studies have shown that the population structure is considerably more complex; hundreds of *T. gondii* genotypes have now been described, with many atypical genotypes in circulation, particularly in Central and Southern America (Shwab et al., 2018) and in wildlife (Su et al., 2006; Miller et al., 2008; Wendte et al., 2011; VanWormer et al., 2014). This finding has led some authors to hypothesise that domestic and wild (sylvatic) *T. gondii* cycles coexist, with archetypal genotypes dominant in anthropised or developed areas, and atypical genotypes dominant in undeveloped areas (Wendte et al., 2011; VanWormer et al., 2014). Despite this, genotypes in the Type II lineage, ToxoDB #1 (clonal Type II) and ToxoDB #3 (variant Type II), are the most common in the world to date, found in cats, humans, livestock, and wildlife, both on land and in the sea (Sibley & Boothroyd, 1992; Sundar et al., 2008; Al-Qassab et al., 2009; Dubey et al., 2009a, 2009b, 2011; Al-Kappany et al., 2010; Parameswaran et al., 2010; Roe et al., 2013, 2017; Shwab et al., 2014; Donahoe et al., 2014, 2015; Cooper et al., 2015, 2016; Brennan et al., 2016).

In New Zealand, a strain of *T. gondii* with PCR-RFLP typing results consistent with variant Type II *T. gondii* has been identified as the cause of fatal disseminated toxoplasmosis in the endangered Hector's dolphin (Roe et al., 2013) and native bird species (Howe et al., 2014). The same genotype was identified in a case of systemic toxoplasmosis in a New Zealand fur seal (Roe et al., 2017), suggesting that this strain is either particularly pathogenic or is widespread in the New Zealand environment (Roe et al., 2017). Genotypes of *T. gondii* in the domestic cat (the only felid species found in New Zealand), however, are not known, and land-sea transmission of *T. gondii* has not been investigated. The study

therefore aimed to: 1) determine the prevalence of oocyst shedding in New Zealand cats; 2) to estimate the annual density of *T. gondii* oocysts shed in New Zealand, and; 3) to examine the diversity of *T. gondii* genotypes in shed oocysts, with the objective of evaluating whether the same variant Type II *T. gondii* previously identified in New Zealand can be traced back to domestic cats (VanWormer et al., 2014), particularly cats sampled from regions adjacent to Hector's dolphin habitat, regions where oocysts may feasibly be transported to the ocean in land-sea run-off.

4.2 Methods

4.2.1 Faecal samples

All cats in the study were opportunistically sampled (*n* = 192) between 2012 and 2015, including stray (*n* = 60) and feral (*n* = 132) cats. For the purposes of this study, stray cats are defined as companion cats (see Chapter 3 for definition) that have been lost or abandoned and that are living as individuals or in a group (colony). Stray cats have many of their needs indirectly supplied by humans and live around centres of human habitation. Stray cats are likely to interbreed with the un-desexed companion cat population. In contrast, feral cats in New Zealand may be defined as cats that live in the wild, they are not stray or owned, they are not socialised, and have no relationship with or dependence on humans (National Cat Management Strategy Group, 2017). Feral cats were recovered from traps maintained as part of Department of Conservation (DOC) predator control programs. Trapped bodies were frozen on site and submitted in batches to Massey University via refrigerated transport. On receipt, bodies were thawed before collection of faeces from the distal large intestine. For some feral cats (*n* = 21), only scats were available. Stray cat carcasses (*n* = 27) and scats (*n* = 33) were provided by local and regional pest control programmes. The stray cases with only scats available were live cats sampled by SPCA animal shelters or the Massey University Veterinary Teaching Hospital (MUVTH) (Table 7). Faecal samples were split into two subsamples where possible. The first (*n* = 192) was processed for zinc sulphate ($ZnSO_4$) flotation, microscopy, and polymerase chain reaction (PCR) on floated oocysts, and the second (*n* = 164) was subjected to whole-faecal PCR (see below). Demographic data (age, sex, region of sampling) was also recorded if available.

4.2.2 Faecal flotation

Faeces were examined for the presence of oocysts using a zinc sulphate ($ZnSO_4$) centrifugation technique. Briefly, 1 g of faeces was thoroughly mixed with 12 ml of 33% $ZnSO_4$ and filtered through a strainer. The strained solution was poured into a 15 ml glass test tube which was filled with $ZnSO_4$ until a slight positive meniscus formed. A coverslip was placed over the tube which was centrifuged at 12,000 rpm for 5 min. The coverslip was then applied to a glass microscope slide and reviewed under a light microscope at 40x magnification for detection of 10 – 12 µm *T. gondii*-like oocysts. Following microscopy, if oocysts were observed, slides were washed with approximately 200 µL of distilled water to collect oocysts for later DNA extraction and PCR.

4.2.3 DNA extraction and nested PCR

DNA was extracted from floated oocysts and from whole faeces using QIAamp® DNA Stool Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A nested PCR (nPCR) was conducted on extracted DNA, amplifying the *dhps* gene of *T. gondii* as described by Aspinall et al. (2002) and in Chapter 2. Ten µl of each PCR product was then run on a 1.5 % agarose gel (Ultrapure agarose, Invitrogen, Carlsbad, CA, USA) with added ethidium bromide at 100 V for 60 min. Select positive samples were purified using a PureLink™ Quick PCR Purification kit (Invitrogen) and submitted for automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA) to confirm genetic sequence, using both the forward and reverse primers. Chromatograms were examined in Geneious 4.8.5 (Biomatters, Auckland, New Zealand) and compared to the other published sequences available from GenBank (Benson et al., 2007) using NCBI Blast. Where sufficient PCR amplification was seen, genotyping of isolates from floated faeces was attempted using multi-locus PCR-RFLP analysis, as described by (Su et al., 2010). Six polymorphic loci were chosen for evaluation: SAG1; SAG2 (5' + 3'); SAG3; GRA6; PK1, and; Apico, to align with previous studies of toxoplasmosis in New Zealand wildlife (Roe et al., 2013, 2017; Howe et al., 2014).

Table 7 Numbers of stray and feral cats sampled in New Zealand by region and whether faeces were available from whole carcasses or as scat only

Island	Region	Stray		Feral		Total
		Carcass	Scat Only	Carcass	Scat Only	
North Island	Northland	-	-	7	-	7
	Auckland	-	-	25	-	25
	Waikato	-	-	1	16	17
	Taranaki	20	-	9	-	29
	Manawatu	7	6	-	-	13
	Wellington	-	27	6	-	33
	Sub-total					**124**
South Island	Nelson	-	-	5	-	5
	Canterbury	-	-	37	5	42
	Otago	-	-	17	-	17
	Southland	-	-	4	-	4
	Sub-total					**68**
	Total					**192**

4.2.4 Estimation of *T. gondii* oocyst loading density

The annual density of *T. gondii* oocyst loading in New Zealand was estimated using the following equation (Dabritz & Conrad, 2010) and previously reported data from New Zealand and overseas (Table 8):

$$\mathbf{D = \{[(O \times \rho O) + E] \times \omega \times \rho T \times K\} / A}$$

where **O** = owned cat population size; **ρO** = proportion of owned cats defecating outside 100% of the time; **E** = feral and stray cat population size; **ω** = annual faecal production per cat of 14 600 g; **ρT** = proportion of cat faeces containing *T. gondii* oocysts; **K** = concentration of *T. gondii* oocysts in cat faeces (e.g. 1.56×10^5 oocysts/g for infections producing 50 million oocysts shed for 8 days) and **A** = land area.

Table 8 Data from New Zealand and overseas used to populate *Toxoplasma gondii* oocyst loading density equation: $D = \{[(O \times \rho O) + E] \times \omega \times \rho T \times K\}/A$ (Dabritz & Conrad, 2010)

Symbol	New Zealand Data
O	1.1 million owned cats[1]
ρO	90 % of which defecate outdoors all the time[2]
E	0.4 – 0.7 million feral and stray cats[1, 3, 4, 5]
ω	14,600 g faeces produced annually per cat[3]
ρT	1.6 % cats are actively shedding (this study)
K	1.56×10^5 oocysts/g of cat faeces[3]
A	263,107 sqkm is the land area of NZ[6]

[1] New Zealand Companion Animal Council 2016; Zito et al., 2019, [2] Farnworth et al., 2010; Hall et al., 2016, [3] Dabritz & Conrad, 2010, [4] Gillies & Fitzgerald, 2005; New Zealand Cat Management Strategy Group, 2016; Farnworth et al., 2013, [5] assuming feral and stray cats account for 25 % - 45 % of the total cat population, [6] Stats NZ, 2015

4.2.5 Statistical analysis: risk factors for shedding

Prevalence of *T. gondii*-like oocyst shedding, confirmed *T. gondii* oocyst shedding, and *T. gondii* DNA in whole faeces was estimated for all cats. Confidence intervals (CI, 95 %) were determined using the Wilson method for binomial proportion data (Newcombe, 1998). To evaluate demographic variables as risk factors for oocyst shedding, both a binomial generalised linear mixed model (GLMM) and a bias-reduced generalised linear model (BRGLM) with logit link functions were fitted to the data, with PCR result from whole faeces as the outcome variable. Univariate analyses were first performed for cat

type (feral, stray), sex, and age (kitten, young adult, adult), using GLM and BRGLM models to estimate crude odds ratios and 95 % CI. All stray cats in the study were sampled in the North Island of New Zealand, so 'island' could not be included as a risk factor in these analyses. Demographic variables associated with shedding ($p \leq 0.20$) were incorporated into multivariable models (VanWormer et al., 2014). An initial GLMM logit was built using region as a random effect, to account for potential correlation between cats sampled from the same location, but the effect of region was considered to be negligible as regression coefficients and their standard errors were not affected. This allowed for the use of bias-reduced logistic regression (BRGLM logit) for the final models, as this method reduces bias in coefficients due to small sample sizes (Firth, 1993; Nemes et al., 2009; Heinze & Puhr, 2010; Pasch et al., 2013). Although not statistically significant in univariate models, age was included in the final multivariate models as a theoretical confounder (Lee, 2014). For multivariate models, adjusted odds ratios with 95 % confidence intervals were estimated to assess the strength of the association between each risk factor and shedding. Cats with missing data were omitted from analyses. Statistical tests were performed in R version 3.4.2 (R Core Team, 2018), using binom v 1.1-1 (Dorai-Raj, 2014), lme4 v 1.1-17 (Bates et al., 2015), and brglm v 0.6.1 packages (Kosmidis, 2011). Missing data were visualised using the package Amelia v 1.7.4 (Honaker et al., 2011) and data availability for demographic variables age and sex is summarised in Table 9.

4.3 Results

From 2012 – 2015, 192 cats were sampled from across New Zealand (see Map 1), comprised of 132 feral (69 %) and 60 stray (31 %) cats. Sex data was available for 130 cats and of these, 65 were male (50 %), and 65 were female (50 %). Age data was available for 89 cats and of these, 12 were kittens (13 %), 27 were young adults (30 %), and 50 were adults (56 %). Oocysts morphologically consistent with *T. gondii* (measuring 10 – 12 µm) were observed in faecal floats from four cats (n = 192), giving a *T. gondii*-like oocyst shedding prevalence of 2.1 % (95 % CI 0.8 – 5.2) (see Map 1). Three of these were confirmed as *T. gondii* by PCR to give a *T. gondii*-confirmed oocyst shedding prevalence of 1.6 % (95 % CI: 0.5 – 4.5). Of the three confirmed *T. gondii* oocyst shedders, one cat was a young adult stray, but the other two were adult ferals. Confirmed oocyst shedders had been sampled from Auckland (Great Barrier

Island), Canterbury, and Manawatu. Thirty-seven out of 164 whole faecal samples (22.6 %, 95 % CI: 16.8 – 29.5) were PCR positive for *T. gondii* DNA. Prevalence results are summarised in Table 10, stratified by cat type.

Table 9 Data availability for demographic variables 'age' and 'sex', for cats with a whole faeces PCR result, stratified by 'cat type' (feral/stray)

Whole Faeces PCR Result	**Positive**		**Negative**		
Cat Type	**Feral**	**Stray**	**Feral**	**Stray**	
Demographic Variable					**Total**
Age	17	3	47	11	58
Sex	21	3	64	22	86
Total	31	6	77	50	164

Table 10 Prevalence of *Toxoplasma gondii* oocysts or DNA in cat faeces. 95 % confidence intervals calculated using Wilson method for binomial proportions.

ShP = shedding prevalence, Prev = prevalence

	Faecal samples	***T. gondii*-like oocysts** Positive	ShP (95 % CI)	**Confirmed *T. gondii* oocysts** Positive	ShP (95 % CI)	**Faecal samples**	**PCR positive for *T.gondii* DNA** Positive	Prev (95 % CI)
Feral cats	132	3	2.3 (0.8 – 6.5)	2	1.5 (0.4 – 5.4)	108	31	28.7 (21.0 – 37.9)
Stray cats	60	1	1.7 (0.3 – 8.9)	1	1.7 (0.3 – 8.9)	56	6	10.7 (5.0 – 21.5)
Total	192	4	2.1 (0.8 – 5.2)	3	1.6 (0.5 – 4.5)	164	37	22.6 (16.8 – 29.5)

4.3.1 Statistical Analysis: risk factors for shedding

Univariate analyses using bias-reduced logistic regression showed that the odds of having *T. gondii* DNA in whole faeces were more than three times higher in feral cats than in stray cats (OR = 3.2, 95 % CI: 1.3 – 9.4, $p = 0.0146$). The presence of *T. gondii* DNA in whole faeces was not significantly associated with age or sex. Interestingly, when age was included as a theoretical confounder in a multivariate BRGLM along with cat type as explanatory variables ($n = 78$), the effect of cat type became insignificant (OR = 1.4, 95 % CI: 0.4 – 8.0, $p = 0.615$). However, this was due to differences in available and missing data, particularly as 42/56 stray cats with a whole faeces PCR result had no available age data. Re-running the univariate analysis for cat type using only animals with available age data showed that cat type was not associated with whole faeces PCR positivity for this subset. Missing data for demographic variables was explored further (Table 9). Using chi-squared or Fisher exact tests where appropriate, stray cats had significantly more missing data for both age and sex ($p < 0.001$), but no significant difference in the proportion of missingness was seen between cats with positive and negative whole faecal PCR results, even amongst stray cats considered alone. There were no missing observations for sampling region.

4.3.2 Genotyping

Genotyping of oocysts isolated from faecal floats was possible for two out of three cats (Cat A and Cat B) with PCR-confirmed samples (summarised in Table 11). Cat A and Cat B were both feral. For the remaining cat (a stray), poor DNA quality and/or poor amplification of the PCR product precluded further analysis. For Cat A, genetic characterisation was successful at five of the six loci tested, with alleles from Type I (GRA6, Apico), Type I or II (SAG2), Type II (L358) and Type III (SAG3). Genetic characterisation was also successful at five of the six loci for Cat B, with alleles from Type I (L358, Apico), Type II or III (SAG1), and Type III (SAG2, SAG3). Comparison of patterns with those in the ToxoDB database (Gajria et al. 2007) showed no match with previously identified genotypes, suggesting the presence of unique genotypes in both cats.

Table 11 Summary of multilocus PCR-RFLP typing for *Toxoplasma gondii* reference strains and for oocysts obtained from New Zealand cats

Isolate/Strain	SAG1	(5' + 3') SAG2	SAG3	GRA6	L358	Apico	Genotype
RH88 (Type I)	I	I	I	I	I	I	ToxoDB #10
PTG (Type II)	II or III	II	II	II	II	II	ToxoDB #1
Variant Type II	II or III	II	II	II	II	I	ToxoDB #3
CTG (Type III)	II or III	III	III	III	III	III	ToxoDB #2
Type 12 (Type A)	II or III	II	II	II	I	I	ToxoDB #4
Type 12 (Type X)	u-1	II	II	II	I	I	ToxoDB #5
Cat A	-	I or II	III	I	II	I	
Cat B	II or III	III	III	-	I	I	

4.3.3 Estimating Oocyst Loading Density

Environmental loading of *T gondii* oocysts in New Zealand was calculated based on prevalence of shedding by feral and stray cats as determined in this study, along with previously reported data, and assumptions about the number of feral and stray cats in the country, as summarised in Table 8. Based on these data and assumptions, the annual environmental burden of oocysts in New Zealand was estimated to be between 5.1 x 10^{13} (51 trillion) and 6.2 x 10^{13} (62 trillion) oocysts per year, meaning 1.9 x 10^{8} oocysts per km^2 (190 oocysts per m^2) to 2.4 x 10^{8} oocysts per km^2 (240 oocysts per m^2) are expected to be shed into the environment annually.

Map 1 Map of New Zealand showing the location of cats sampled which were found to be shedding *T. gondii*-like oocysts by faecal flotation, stratified by cat type (stray or feral). Yellow dots represent feral cats. Blue dots represent stray cats. Gray shading shows regions from which cats were sampled.

4.4 Discussion

4.4.1 Genotypes

Three cats in the present study (*n* = 192) had *T. gondii* oocysts in their faeces. Partial genotyping using PCR-RFLP was possible for two of the three isolates, revealing what appeared to be two unique, recombinant genotypes, each with a combination of alleles normally seen in the three archetypal clonal lineages of *T. gondii* (Types I, II, and III) (Belfort-Neto et al., 2007; Dardé, 2008; Parameswaran et al., 2010). These results suggest that novel strains of *T. gondii* exist in New Zealand and indicate that a diversity of genotypes may be circulating in the country. Although the number of isolates genotyped in this study was very small, this hypothesis is supported by results from another New Zealand study (Mirza et al., 2017) that recently found evidence of an additional recombinant genotype of *T. gondii*, which had a unique combination of Type I and Type II alleles, in tissues from six endemic raptors. No evidence of toxoplasmosis was found in the raptors and the potential infection risk of these novel genotypes for intermediate hosts in New Zealand is yet unknown. Further genotyping, however, ideally using murine bioassay, PCR, TA cloning, and sequencing is required to confirm the presence of unique recombinant and atypical genotypes, and to rule out the possibility that the cats had mixed infections with more than one *T. gondii* genotype (Dubey et al., 2009b).

A specific objective of this study was to determine whether the same variant Type II genotype linked to fatal disseminated toxoplasmosis in Hector's dolphins was present in cat faeces. This genotype was not detected in the study cats. A number of reasons can be put forward to explain why it was not found, including sample size, type of cat included in the study, and type of sample tested. The proportion of cats actively shedding oocysts at any one time is relatively low compared with the proportion infected (Dubey, 2004, 2016), which would significantly reduce the likelihood of finding a particular *T. gondii* genotype. Considering the sample size of this study, it was therefore unlikely that variant Type II *T. gondii* would be detected, as oocyst shedding prevalence was only 1.6 %, and only a small number of isolates were available for genotyping. In order to increase the likelihood of finding variant Type II, as well as increasing the number of cats sampled, future research could genotype *T. gondii* isolates from

fresh cat tissues as well as oocysts, following California-based feline studies (Miller et al., 2008; VanWormer et al., 2014). This is because the proportion of cats infected with *T. gondii* at any given time is much higher than the proportion actively shedding and tissue cysts persist in infected host tissues, perhaps for life (Dubey, 2016).

Beyond this, variant Type II may not have been detected in this study because genotyping was only possible for feral cat oocysts. Prior research suggests that atypical or recombinant genotypes are more common in felids that live in wild, undeveloped areas, whereas the archetypal *T. gondii* strains, including variant Type II, are more prevalent in felids that live in developed areas (Herrmann et al., 2010; VanWormer et al., 2014; Verma et al., 2017; Jiang et al., 2018). It is therefore possible that an independent sylvatic transmission cycle maintained by feral cats, in which recombinant or atypical genotypes predominate, coincides with a domestic Type II-based transmission cycle in New Zealand, maintained by companion and/or stray cats, as occurs in California (VanWormer et al., 2014).

4.4.2 Shedding Prevalence

In this study, the proportion of cats shedding *T. gondii* oocysts was estimated to be 1.6 % (95 % CI: 0.5 – 4.5; *N* = 192). Although low, this shedding rate was within expected limits. The reported prevalence of *T. gondii* or *T. gondii*-like oocysts in domestic cat faeces worldwide ranges from 0 – 34 % (summarised by Dabritz & Conrad, 2010; Dubey et al., 2013; Chemoh et al., 2016; Veronesi et al 2017), but is typically <2 % where the estimate is based on flotation and light microscopy (e.g., Miró et al., 2004; Pena et al., 2006; Dabritz et al., 2007; Karatepe et al., 2008; Schares et al., 2008, 2016; Herrmann et al., 2010; Mancianti et al., 2010; Berger-Schoch et al., 2011; Jokelainen et al., 2012). Serological studies indicate widespread exposure to *T. gondii* in most domestic cat populations (see Chapter 3; Dabritz & Conrad, 2010) but the proportion of cats actively shedding oocysts is expected to be low because oocysts are only shed in cat faeces over relatively short periods. The probability of sampling a cat which is actively shedding will be small, unless high risk animals can be specifically targeted.

The proportion of cats actively shedding oocysts in New Zealand as estimated in this study is likely to be an underestimate. This is because flotation and light microscopy-based methods have a relatively low sensitivity compared with alternatives, particularly bioassay or PCR of DNA extracted from whole faeces (Dabritz et al., 2007; Salant et al., 2010; Dubey et al., 2013). Two separate studies suggest that the analytical sensitivity of flotation coupled with light microscopy ranges between 250 – 1000 oocysts per gram of faeces (Dabritz et al., 2007; Jones & Dubey, 2010). Microscopy techniques could therefore miss low level shedding, which may occur at the tail ends of the shedding period, in cats that are infected through ingestion of oocysts rather than tachyzoites or bradyzoites in tissue cysts (Dubey & Frenkel, 1976; Dubey, 1996; Salant et al., 2007), and possibly in cats that re-shed after first infection (Dubey, 1995; Zulpo et al., 2018).

Indeed, in this study whole-faecal PCR prevalence was 22.6 % (95 % CI: 16.8 – 29.5; *n* = 164), which was considerably higher than faecal float prevalence. This whole faecal PCR result was relatively high compared with some overseas studies (Salant et al., 2007, 9%, *n* = 122; Lilly & Wortham, 2013, 6 %, *n* = 49; Chemoh et al., 2016, 4.7 %, *n* = 254; Davis et al., 2018, 5 %, *n* = 60) and comparable to others (Mancianti et al., 2010, 16 % *n* = 50; Veronesi et al., 2017, 20.5 %, *n* = 78; Davis et al., 2018, 22.2 %, *n* = 9). Care needs to be taken with the interpretation of whole faecal PCR results, however. The higher proportion of cats with *T. gondii* DNA in their faeces than observed using microscopy alone could be due to the increased sensitivity of PCR relative to microscopy; detection of sexual stages of *T. gondii* within sloughed intestinal epithelial cells (Dubey & Frenkel, 1972); or to the presence of asexual stages of *T. gondii* in the intestine of re-infected cats that are already immune to *T. gondii*, and which will not re-shed (Davis & Dubey, 1995; Mancianti et al., 2010). Furthermore, recent work has shown that highly sensitive molecular methods, namely quantitative PCR targeting multi-copy markers in the *T. gondii* genome, may detect *T. gondii* DNA from bradyzoites in prey ingested by cats, in the absence of oocysts (Poulle et al., 2016) . Overall, results here highlight the difficulties in determining true oocyst shedding prevalence using microscopy techniques, even when coupled to PCR. Nevertheless, results clearly

demonstrate that *T. gondii* is present in feral and stray cats, and possibly their prey, in the regions sampled across New Zealand.

4.4.3 Environmental loading with *T. gondii* oocysts

The annual environmental burden of oocysts in New Zealand was calculated to be between 1.9 x 10^8 oocysts per km^2 (190 oocysts per m^2) and 2.4 x 10^8 oocysts per km^2 (240 oocysts per m^2). Prior studies have attempted to estimate the environmental burden of oocysts overseas, using the same assumptions about the density of oocysts per gram of cat faeces, and faecal production (Sousa et al., 1988; Dabritz et al., 2007; Dabritz & Conrad, 2010). The density estimate calculated in this study is over three times lower than that of Dabritz & Conrad (2010), who calculated that for the USA, 779 – 1728 oocysts per m^2 would be disseminated into the environment, assuming that feral cats comprised either 25 % or 45 % of the total cat population. The estimate for New Zealand given here is also over 19 times lower than the 4671 oocysts per m^2 environmental burden reported for three coastal communities in California, USA, which had approximately 10,000 owned and feral cats (Dabritz et al., 2007), and over 40 times lower than the estimated 9650 – 38700 oocysts per m^2 for 50 – 200 cats that lived in a 7.7 ha community in Panama (Sousa et al., 1988).

The disparity between the New Zealand estimate and those overseas could reflect real differences in oocyst load between locations, perhaps due to differences in cat population sizes (oocyst shedding rates in the aforementioned studies were lower than our New Zealand estimate). For example, although latest figures show that the proportion of households owning a cat is higher in New Zealand (44 %, New Zealand Companion Animal Council, 2016) than the USA (38 %, American Pet Products Association, 2018), the average number of cats per household is greater in the USA (2.0 versus 1.5 in New Zealand). There is also a high level of uncertainty surrounding feral and stray cat population sizes in New Zealand (Farnworth et al., 2013). In contrast, the disparity could reflect the size of the area under consideration, an argument put forward by Dabritz & Conrad (2010). For example, whilst Dabritz & Conrad (2010) used cat population estimates for the whole of the USA in their calculations, land area

was represented by Metropolitan Statistical Areas (MSAs), which only constitute approximately 20 % of the USA, whereas the entire land area of New Zealand was used in this study. When the estimate of Dabritz & Conrad (2010) was re-calculated using 100 % of the US land area (9.834 x 10^6 km^2) rather than MSAs (1.83 x 10^6 km^2), a new density of 1.44 x 10^8 oocysts per km^2 – 2.47 x 10^8 oocysts per km^2 was given, comparable to the estimate for New Zealand. It is also important to consider that the actual oocyst burden in New Zealand, as elsewhere, is likely to be most highly concentrated around cat defecation sites (Afonso et al., 2008), and also within feral cat ranges, around stray cat colonies or households with a high proportion of cat ownership and outdoor cats (Dabritz et al., 2006; Afonso et al., 2008; Dabritz & Conrad, 2010).

4.4.4 Demographic risk factors for *T. gondii* shedding

For the domestic cat, risk factors previously linked to oocyst shedding include management status (feral, stray, or owned) (VanWormer et al., 2013a; Davis et al., 2018) and age (VanWormer et al., 2013a). An in-depth examination was not possible here, as the study was primarily designed to detect and genotype *T. gondii* oocysts. However, univariate analysis showed that feral cats were more than three times as likely to be shedding *T. gondii* than stray cats (OR 3.2; $p = 0.01$), and a similar pattern has previously been observed overseas (VanWormer et al., 2013a, 2013b; Davis et al., 2018). For example, VanWormer et al. (2013a) found that unmanaged feral cats (and bobcats) were over 13 times more likely to be shedding *T. gondii*-like oocysts than managed feral (stray) cats in California, USA ($p = 0.04$). Higher shedding in feral cats than strays in New Zealand may be attributed to differences in diet, the frequency and type of prey eaten, as well as the prevalence of *T. gondii* infection in different prey species (Afonso et al., 2006; VanWormer et al., 2013a). Predation is considered to be the major cause of *T. gondii* infection in cat populations (DeFeo et al., 2002; Afonso et al., 2007; Simon et al., 2018) and whereas feral cats in New Zealand subsist predominantly through predation (Wilson, 2004; Gillies & Fitzgerald, 2005), stray cats may consume fewer potential intermediate hosts due to access to alternative food sources, such as commercial cat food and scraps.

It is important to remember, though, that univariate results were based on the presence of *T. gondii* DNA in whole faeces, which may not be the best proxy for true levels of oocyst shedding (discussed above). Using faecal floatation and microscopy techniques, two feral cats (1.5 %, 95 % CI: 0.4 – 5.4, *n* = 132) and one stray cat (1.7 %, 95 % CI: 0.3 – 8.9, *n* = 60) were found to have *T. gondii* oocysts in their faeces, suggesting that feral and stray cats may actually have similar oocyst shedding prevalences, although statistical analysis was precluded by small shedding numbers. If this is the case, higher odds of *T. gondii* DNA in cat faeces may better reflect greater consumption of infected prey (Poulle et al., 2016). Nevertheless, diet may also impact immune status, exposure to *Isospora felis (*another common feline parasite) and new strains of *T. gondii*, factors which have been found to result in oocyst re-shedding in previously infected cats under experimental conditions (Chessum, 1972; Dubey & Frenkel, 1974; Dubey, 1995; Lukesova & Literák, 1998; Zulpo et al., 2018). Feral cats consuming greater quantities of infected prey would be exposed to new *T. gondii* strains and *I. felis* more frequently, increasing their risk of repeat shedding and their observed shedding prevalence (VanWormer et al., 2013a).

Age was included as a potential risk factor and theoretical confounder in this study because oocyst shedding has been observed by some researchers to occur predominantly when weaned kittens are first exposed to prey infected with *T. gondii* (Ruiz & Frenkel, 1980; Dubey & Beattie, 1988; Salant et al., 2007). Kittens are also documented to shed higher number of oocysts following primary infection than older cats (Ruiz & Frenkel, 1980; Dubey & Beattie, 1988; VanWormer et al., 2013b). As such, the odds of kittens shedding *T. gondii* may be greater than the odds for young and older adults. This pattern was not confirmed amongst the study cats, however. Considering whole faecal PCR results, the proportion of PCR positive young adult cats was higher than kittens, and the proportion of PCR positive adults was lower than both other age categories (results not shown). This suggests that there may be a trend with higher shedding prevalence in younger cats but univariate analysis showed no significant effect of age on detection of *T. gondii* DNA in faeces. Limitations of the study should be considered when interpreting results. Particularly, there was a problem of missing age data, which may have biased the estimate, and introduced a loss of statistical power and precision. Also, misclassification bias may have

impacted the observed association, as cat ages were not exact. Again, univariate results were based on the presence of *T. gondii* DNA in whole faeces which may not accurately reflect oocyst shedding. Of the three cats found to be shedding oocysts by faecal float and PCR, none were kittens. One was a young adult stray, and two were adult ferals. Although numbers were too small to permit statistical analysis, faecal float results show that oocyst (re-)shedding also occurs in older cats in New Zealand. Results are in line with those of previous studies in which oocyst shedding was detected in naturally-infected adult domestic cats (1 – 18 years old) from diverse geographical locations (Schares et al., 2008; Herrmann et al., 2010; Berger-Schoch et al., 2011; VanWormer et al., 2013a). Overall, results suggest that feral and stray cats in New Zealand are exposed to *T. gondii* at an early age, and that adult cats are also a source of environmental contamination with *T. gondii* oocysts.

Although it was not possible to include sampling region as a risk factor in this study, cats shedding *T. gondii* oocysts were sampled from both the North and South Island of New Zealand. Furthermore, cats positive for *T. gondii* DNA in whole faeces were sampled from each of the ten regions included in the study. *Toxoplasma gondii* is therefore widespread in feral and stray cats in New Zealand and, presumably, their prey. Further research is needed to assess prevalence of *T. gondii* infection in prey species, and to determine oocyst shedding prevalence in companion cats, which may also contribute to oocyst loading in New Zealand.

4.5 References

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5 Investigating clinical toxoplasmosis in domestic cats (*Felis catus*) in New Zealand

5.1 Introduction

Toxoplasma gondii is one of the world's most common apicomplexan parasites and the causative agent of toxoplasmosis. *T. gondii* is able to infect most, if not all, species of birds and mammals as intermediate hosts, but can only complete its lifecycle in the cat, making felids the definite host (Dubey, 2016). As such, felids are the only animals that can shed oocysts in their faeces after infection (Frenkel et al., 1970; Di Genova et al., 2019), although intermediate hosts, including cats, can harbour infective tissue cysts which may persist for life (Dubey et al., 2009; Elmore et al., 2010). Post-natal infection through consumption of infected intermediate hosts is thought to be the typical transmission route for cats, but congenital infection can also occur (Dubey et al., 2009; Elmore et al., 2010). Serological surveys conducted worldwide have shown that *T. gondii* infection is prevalent in wild, feral, and domestic cat populations (e.g., Afonso et al., 2006; Dabritz et al., 2007; Dubey & Jones, 2008; Dabritz & Conrad, 2010; Jokelainen et al., 2012; VanWormer et al., 2013; Chapter 3), with a global seroprevalence estimate of 30 – 40 % for domestic cats (Dubey & Beattie, 1988; Vollaire et al., 2005; Haddadzadeh et al., 2006; Liu et al., 2015). Despite evidence of widespread exposure to *T. gondii* in domestic cats, most infections are subclinical and chronic, as is the case for the majority of host species (Elmore et al., 2010; Must et al., 2017). Only a small proportion of cats develop severe clinical disease, but toxoplasmosis is still considered to be an important cause of feline mortality, particularly for cats with suppressed immune systems (Henriksen et al., 1994; Jokelainen et al., 2012).

Clinical signs of feline toxoplasmosis are varied but commonly include anorexia, lethargy, and dyspnoea due to pneumonia, as well as fever, icterus, ocular inflammation, abdominal discomfort and neurologic abnormalities (Hirth & Nielsen, 1969; Lappin et al., 1989; Dubey & Carpenter, 1993; Vollaire et al., 2005; Dubey & Prowell, 2013), as summarised by Dubey et al. (2009). Affected cats can also die

suddenly with no obvious clinical signs. Ante-mortem serological testing can suggest a diagnosis of toxoplasmosis in cases with high titres, but confirmation of generalised disease due to *T. gondii* requires histology to demonstrate consistent lesions, along with immunohistochemistry (IHC), polymerase chain reaction (PCR), or electron microscopy to confirm presence of the agent within lesional tissues. Characteristic lesions of toxoplasmosis in cats after experimental or natural infection have been well described (e.g. Hirth & Nielsen, 1969; Parker et al., 1981; Davidson et al., 1993; Dubey & Carpenter, 1993; Henriksen et al., 1994; McConnell et al., 2007; Spycher et al., 2011; Jokelainen et al., 2012; Nagel et al., 2013), and are predominantly seen in the lung, liver, central nervous system (CNS), eyes.

As severe disease is unusual in cats, there has been a growing interest in understanding the predisposing factors of clinical infection. Proposed factors include the age of the cat, concurrent infections, and immunosuppression. Congenitally infected kittens are more likely to develop fatal disseminated toxoplasmosis than cats which acquire *T. gondii* post-natally (Dubey & Carpenter, 1993; Dubey et al., 2009), and concomitant illness or immunosuppression may increase susceptibility to severe systemic disease, although healthy adult cats are also affected (Dubey & Carpenter, 1993; Spycher et al., 2011; Jokelainen et al., 2012; Nagel et al., 2013). The severity of clinical disease in some infected hosts also appears to be influenced by *T. gondii* genotype (Sibley & Boothroyd, 1992; Dardé, 2008; Shapiro et al., 2019), but it is not yet clear to what extent *T. gondii* strain type plays a role in the pathogenesis of clinical feline toxoplasmosis (Spycher et al., 2011; Jokelainen et al., 2012; Dubey & Prowell, 2013; Nagel et al., 2013; Brennan et al., 2016).

New Zealand provides a unique research context because the country has few native mammals, and no felid species except the domestic cat (*Felis catus*) (King, 1990). The domestic cat, however, is the most popular companion animal for New Zealanders (New Zealand Companion Animal Council, 2016) and New Zealand is home to large populations of owned, stray, and feral cats (Farnworth et al., 2013). Furthermore, *T. gondii* appears to be prevalent in New Zealand; the parasite may cause 20 – 30 % of

ovine abortions in the country, documented as the second most common abortifacient of sheep (West, 2002; Vet Services Hawke's Bay 2015). One particular genotype of *T. gondii* - variant Type II (ToxoDB #3) - has also been associated with fatal cases of toxoplasmosis in certain species of endemic bird (Howe et al., 2014) and marine mammal (Roe et al., 2013, 2017) in New Zealand. Cases of feline toxoplasmosis in New Zealand have not previously been reported on in detail, and it is not known whether variant Type II *T. gondii* is also virulent for local domestic cats. The aims of this study were therefore to describe clinical cases of toxoplasmosis in New Zealand domestic cats, and to identify the *T. gondii* genotype(s) present.

5.2 Methods

5.2.1 Case selection and re-examination

The Pathobiology Group database at the School of Veterinary Science, Massey University, was searched for feline cases submitted between 1 August 1991 and 31 July 2017, that included the keyword 'toxoplasma' or 'toxoplasmosis'. Full records for each case with either of these keywords were retrieved and those with a final diagnosis of toxoplasmosis, as determined by the evaluating pathologist, were chosen for further investigation. Clinical history, original pathology reports, and histological tissue sections were re-examined by an individual veterinary pathologist for consistency (see Table 12). Tissue samples had previously been routinely fixed in 10 % buffered formalin, embedded in paraffin (formalin fixed paraffin embedded (FFPE) tissues), and sectioned at 4 µm for microscopic examination. All sections were stained with haemotoxylin and eosin (H&E) and with special stains (Gram, Young's fungal, Giemsa, Perl's, periodic acid-Schiff (PAS), Congo red, and Masson's trichrome) where indicated. For each case that had FFPE tissues available, blocks were selected for immunohistochemical analysis and molecular characterisation by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. Selection was based on the presence of lesions consistent with toxoplasmosis (Hirth & Nielsen, 1969; Parker et al., 1981; Davidson et al., 1993; Dubey & Carpenter, 1993; Henriksen et al., 1994; McConnell et al., 2007; Spycher et al., 2011;

Jokelainen et al., 2012; Dubey & Prowell, 2013; Nagel et al., 2013), in conjunction with intralesional protozoan-like structures on H&E sections.

5.2.2 Immunohistochemistry for *T. gondii*

Immunohistochemistry (IHC) for *T. gondii* was carried out on a selection of FFPE tissues from all cases with a diagnosis of toxoplasmosis, as described by Roe et al. (2013) and in Chapter 2, with the following modifications. Briefly, endogenous peroxidase activity was blocked using 3 % hydrogen peroxide in methanol for 10 min at room temperature and was followed by antigen retrieval in 0.1 % trypsin/calcium chloride (pH 7.6). Blocking of non-specific binding sites was performed using 0.1 % gelatin in phosphate buffered saline (PBS 1X, pH 7.2). Primary antibody (polyclonal caprine anti-*T. gondii* antibody; VMRD, Pullman, WA, USA) diluted to 1:3000 in 0.1 % gelatin was then applied to the slides which were kept refrigerated at 4 ^{o}C overnight. Additionally, one cat tissue section per case was incubated with polyclonal caprine anti-*Neospora caninum* antibody as the primary antibody (VMRD) diluted to 1:3000 in 0.1 % gelatin. Slides were then washed with 0.2 % Tween-20 in PBS before incubation with secondary antibody (biotinylated donkey anti-goat antibody; Rockland Immunochemicals Inc., Gilbertsville, PA, USA) diluted to 1:500 in 0.1 % gelatin with 0.5 % Tween-20 for 60 min at room temperature. Visualisation was performed using the Vectastain Universal Elite kit (Vector Laboratories; Burlingame, CA, USA) and the Immpact DAB Peroxidase Substrate (Vector Laboratories), according to the manufacturer's instructions. A positive antibody control was included in each batch of slides using sequence-confirmed *T. gondii* positive cat tissue sections. A negative control was also included which comprised the omission of anti-*T. gondii* primary antibody on known positive cat tissue sections.

5.2.3 Molecular characterisation of *T. gondii* parasites

For each case, the tissue with the largest number of immunopositive organisms was selected for molecular analysis. DNA was extracted in triplicate from 10 µm slices of these FFPE tissues (Extraction 1 – 3) using a Qiagen DNeasy Kit (Qiagen, Valencia, California, USA), following manufacturer's

instructions, except that DNA was eluted in 100 µl AE buffer in Extraction 2 and 3. All samples were screened in duplicate for the *dhps* gene of *T. gondii* using a nested PCR assay described by Aspinall et al. (2002) (nPCR *dhps*). In addition, using DNA from Extraction 1 only, PCR amplification of the *T. gondii* B1 gene was performed as described by Grigg & Boothroyd (2001) (nPCR B1) (see Appendix 5) and of the apicomplexan phylum internal transcribed spacer region 1 (ITS1), following the protocol described by Payne & Ellis (1996) (PCR apicomp). DNA was then extracted from a single 10 µm sample of all remaining tissues per case (Extraction 4) and screened once using each of the PCR assays. To confirm successful amplification, 10 µl of the final PCR product was run on a 1.5 % agarose gel containing SYBR Safe (Thermo Fisher Scientific, Boston, MA, USA). A known *T. gondii* isolate (incomplete strain S48, Toxovax®, MSD Animal Health, New Zealand), confirmed by sequencing, was used as a positive control. DNA extraction controls and water blanks were included as negative controls. For cat samples, presence of a band of expected size was taken as qualitative evidence of successful amplification of *T. gondii* or apicomplexan DNA. Where sufficient amplification was seen from *T. gondii*-specific PCRs, genotyping was attempted using multi-locus PCR-RFLP analysis, as described by Su et al. (2010) and Shapiro et al. (2015). Eight loci were chosen for evaluation: SAG1; SAG2 (5' + 3'); SAG3; GRA6; PK1; Apico; L358, and; B1, to align with previous studies of toxoplasmosis in New Zealand wildlife (Roe et al., 2013, 2017; Howe et al., 2014). Where sufficient amplification was seen from the PCR apicomp, PCR products were purified using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA) and submitted to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems). Sequences were analysed using Geneious software 8.1.5 (Biomatters) and compared with reference sequences for *T. gondii* using a BLAST search (GenBank database, NCBI).

5.2.4 Transmission Electron Microscopy

For each case, one FFPE tissue was selected and processed for transmission electron microscopy (TEM). Tissues selected were those containing the largest number of immunopositive organisms as determined by IHC. Cat 4 was immunonegative so the tissue selected for this case was that which had visible *T. gondii*-like organisms on H&E. Paraffin was melted from the FFPE tissues by placing the blocks in the 60

°C wax bath of a HistoStar Embedder (ThermoFisher, Boston, MA, USA). Specific areas of interest were located on H&E and IHC slides before excision using a new biopsy punch for each case (4 mm, SMI AG, St Vithy, Belgium). Excised areas were then divided into four or more smaller fragments. Fragments were placed into cassettes (one cassette per case) which were submerged in a beaker filled with xylene. The beaker was placed on a stirrer and agitated for a minimum of 30 mins, after which the xylene was removed and replaced with fresh xylene and agitated for another 30 mins (+). Xylene was then replaced with absolute alcohol followed by agitation for 30 min. The latter step was repeated twice more, changing the absolute alcohol each time. Following this, the absolute alcohol was replaced with 95 % alcohol and the samples agitated for 30 min (+); this step was repeated once more changing the 95 % alcohol. Next, the 95 % alcohol was replaced with 70 % alcohol and the sample was agitated for 30 min. This step was repeated once more. Then, the samples were rinsed in running tap water before being placed into primary EM fixative (Modified Karnovsky's Fixative (3 % Gluteraldehyde (Merck) (v/v) 2 % Formaldehyde (w/v) in 0.1M Phosphate Buffer (pH 7.2)), and were allowed to fix for at least 8 hours. The samples were then buffer washed in 0.1M phosphate buffer (pH 7.2) three times for 10 min each then post fixed in 1% OSO4 in 0.1M phosphate buffer for 1 hour at room temperature. They were then buffer washed again (as above) then dehydrated through a graded acetone series (25 %, 50 %, 75 %, 95 %, 100 %) for 10 – 15 min each followed by two changes of 100 % acetone for one hour each. The samples were placed into 50:50 resin:acetone and placed on the stirrer overnight followed by replacement with fresh 100% resin (Procure 812, ProSciTech Australia) for 8 hrs on the stirrer. This step was repeated twice more (overnight in 100 % resin, 8 hours in 100 % resin) before the samples were embedded in moulds with fresh resin and cured in a 60°C oven for 48hrs. Light microscope sections were cut at 1 micron using a glass knife on the ultramicrotome (Leica EM UC7, Germany) and heat fixed onto glass slides. These were stained with 0.05 % Toluidine Blue for approximately 12 seconds and viewed under the light microscope. The block was then trimmed down to the selected area and cut using a Diamond Knife (Diatome, Austria) at 100nm. These were stretched with chloroform and mounted on a grid using a Quick Coat G pen (Saiko, Japan). Grids were stained in Saturated Uranyl Acetate in 50% Ethanol for 4.5 min, washed with 50 % ethanol and MilliQ water and then stained in Lead Citrate (Venable and Coggeshall, 1965) for a further four and a half minutes. This was followed by

a wash in MilliQ water. Samples were viewed using a FEI Tecnai G^2 Spirit BioTWIN Transmission Electon Microscope (Czech Republic).

5.2.5 Final interpretation of test results

A cat was given a definitive diagnosis of toxoplasmosis if each of the following conditions were met:

1) histological lesions consistent with toxoplasmosis

2) protozoal organisms in tissues were immunopositive by *T. gondii* IHC

3) DNA extracted from lesional FFPE tissues was positive on at least one PCR and confirmed as *T. gondii* by one or more of:

    a. PCR-RFLP genotyping

    b. Sequencing

    c. TEM

5.3 Results

Between August 1991 and July 2017, 3332 feline cases were submitted to the Pathobiology Group at Massey University for evaluation. Of these, 37 cases included the keywords 'toxoplasma' or 'toxoplasmosis', and nine of these cats had been given a final diagnosis of toxoplasmosis, based on characteristic histological lesions with the presence of protozoal organisms on H&E sections. FFPE tissues were available from six of these cases (Cats 1 – 6) for further investigation. Tissues had been collected either during surgery (Cat 1; stomach biopsy) or at necropsy (Cats 2 – 6; full representative tissue sets).

5.3.1 Case histories

Details are summarised in Table 12. The most common presenting signs were anorexia and vomiting, followed by weight loss, diarrhoea, dyspnoea, jaundice (Cats 4 and 5) and anisocoria (Cats 4 and 5). Toxoplasmosis was diagnosed ante-mortem in Cat 2, which had a serum antibody titre to *T. gondii* of

1:1024, and a severe interstitial pattern on lung radiographs. Three cats had been administered potentially immunosuppressive drugs. The first cat (Cat 1) was hyperthyroid and had been treated with carbimazole. The second (Cat 2) had previously been administered an unspecified steroid for feline asthma/bronchial disease and the third (Cat 4) had a history of inflammatory bowel disease (IBD), treated with prednisone and prednisolone, along with clinical and histological evidence of chronic renal failure. Dose rates for these drugs were not recorded. Four of the study cats were adults (Cats 1, 2, 4, 5) including three that were known to between 10 and 13 years old (Cats 1, 4, 5). The age of Cat 2 was not specified. The remaining two cases were eight-week-old kittens. One of the kittens came from a household where eight other kittens had died within the previous two weeks, but its littermates and dam remained healthy, while the other was a shelter kitten that became ill after it had been vaccinated and spayed, and was euthanised.

5.3.2 Gross, cytological, and histological lesions

Gross, cytological, and histopathological findings are summarised in Table 12. Cat 1 was presented to the Veterinary School Teaching Hospital for investigation of chronic weight loss, vomiting and anorexia. On endoscopic examination, the stomach wall was severely thickened. Stomach biopsies were collected for cytological and histological evaluation. Cytology showed a mixed inflammatory infiltrate. Histology revealed severe pyloric infarction, with prominent granulation tissue and mixed cell inflammatory infiltrate in the adjacent stomach wall. Large numbers of small, mainly spherical, organisms were seen throughout, often in clusters of one hundred or smaller aggregates containing 2 – 20 organisms.

Cats 2 – 6 were presented dead to the Veterinary School postmortem service for necropsy examination. Gross lesions were found in a number of organs but were often non-specific or were related to concurrent disease processes. For the purposes of this study, gross lesions that were due to concurrent diseases (i.e. not due to toxoplasmosis) were not described further. The most common grossly affected organ was the liver. Cats 2, 3, and 5 had multiple 1 – 4 mm pale foci on the surface or throughout the

parenchyma, and cat 5 had a mildly enlarged liver with irregular red mottling on the surface. Two cats had gross lung lesions, one with diffuse white/pink mottling and copious mucus within the airways, (Cat 2), and the other with lungs that were red, heavy and oedematous (Cat 3). Cytological preparations from the lung of Cat 5 showed many alveolar macrophages with intracytoplasmic encysted protozoal bradyzoites, and free tachyzoites. Gross lesions subsequently attributed to toxoplasmosis were also present in the brain (swelling with flattening of the gyri; Cat 3), and pancreas (enlarged and pale with multiple areas of peri-pancreatic fat necrosis; Cat 5).

Histopathological lesions of toxoplasmosis in these 5 cats were predominantly seen in the liver (Cats 2, 3, 5, and 6), brain (Cats 3 – 6), and lung (Cats 2, 3, 5, 6). Liver lesions consisted of multi-focal to coalescing areas of hepatocellular degeneration and/or necrosis with an associated mild-moderate inflammatory infiltrate. Rare protozoan tissue cysts were detected within these lesions in two cases (Cats 3 and 5) and structures resembling free tachyzoites were observed in one case (Cat 3). In the brain of cats 3, 5, and 6, there were multifocal areas of gliosis and/or necrosis accompanied by a mild-moderate inflammatory infiltrate. Lesions tended to be distributed throughout the parenchyma of the cerebrum but were also present in the cerebellum of Cat 3. Brain lesions were associated with occasional tissue cysts in each of these cats. Cat 6 also had moderate numbers of mononuclear cells within the subarachnoid space, particularly surrounding blood vessels. Cat 4 had a single area of gliosis in the white matter of the cerebrum. Adjacent to this was a small cluster of 10 – 12 structures consistent with protozoal cysts, with no accompanying inflammation.

Of the five cats examined, four (Cats 2, 3, 5, 6) had moderate to severe interstitial pneumonia consistent with toxoplasmosis, associated with marked Type II pneumocyte hyperplasia and inflammatory infiltrate, necrosis, haemorrhage, and oedema. Occasional free tachyzoites were detected. Ocular lesions were reported in three cases (Cats 2, 4, and 5), characterised by choroiditis. For Cat 5, inflammation of the choroid and sclera was associated with rare tissue cysts. No protozoal organisms were seen in sections of eye from Cats 2 and 4. Pancreatic lesions attributed to

toxoplasmosis were seen in sections from Cats 2 and 5. Lesions in the pancreas of Cat 2 were limited to several small foci of inflammation in the peri-pancreatic fat. In Cat 5, however, there was moderate atrophy, fibrosis, and hyperplasia of exocrine tissue with rare protozoal tissue cysts, with inflammation and necrosis of the peri-pancreatic fat. Myocarditis was recorded in Cats 2 and 3, with occasional small foci of inflammation and necrosis. Occasional tachyzoites were observed in Cat 3, both free and within myocytes. In the spleen of Cat 3, there was significant, diffuse haemorrhage and necrosis. Tachyzoites and tissue cysts were observed throughout. *Toxoplasma*-like lesions and parasites were detected in the bladder of Cat 5. Tissue cysts and tachyzoites were present within myocytes of the muscular layer of the bladder wall accompanied by a mild inflammatory infiltrate and myofibre necrosis.

5.3.3 Immunohistochemistry

A total of 83 % (5/6) of the cats evaluated were positive for *T. gondii* by IHC (see Table 13). Cat 4 was immunonegative on all tissues tested. Three of the five immunopositive cats were positive for *T. gondii* in all tissues tested (Cats 1, 3, 6). The remaining two cats (Cats 2 and 5) were immunopositive for all tissues except the eye in which melanin granules obscured immunostaining. Positive tissues demonstrated immunoreactivity to anti-*T. gondii* antibody, as shown in Figures 9 – 11. In these tissues, immunostained parasites were observed, including crescent-shaped to spherical tachyzoites and small round cysts containing bradyzoites. IHC therefore confirmed that the protozoal structures observed on the selected H&E stained sections were *T. gondii* tissue cysts and tachyzoites, except for Cat 4. In this case, no protozoan-like structures were evident at the location corresponding to those seen in the H&E slide, and the tissue was immunonegative. For some tissue sections, IHC enabled visualisation of organisms that were not apparent on H&E stained sections (see Table 13). Immunohistochemical staining for *Neospora caninum* was conducted for each case in the IHC study because lesions due to *N. caninum* infection in cats are histologically similar to those of feline toxoplasmosis (Dubey et al. 1990). In one case (Cat 2), protozoal organisms in the lung were immunopositive for both *T. gondii* and *N. caninum*, (Figure 11A, B); however, molecular characterisation by PCR-RFLP and sequencing confirmed they were *T. gondii* rather than *Neospora* spp. (see Tables 13 and 14, and below).

5.3.4 Molecular characterisation

Results from the molecular analyses are summarised in Tables 13 and 14. Triplicate DNA extractions from tissues with the highest number of IHC-positive organisms (Extractions 1 – 3) were first screened for *T. gondii* DNA using the nPCR *dhps*. Although *Toxoplasma* parasites were abundant and easily visible on IHC sections, only the lung tissue from Cat 2 and the brain from Cat 4 were PCR positive for *T. gondii* DNA, and Cat 4 brain was only weakly positive. Remaining tissues from each case (Extraction 4) were screened once using the nPCR *dhps* and Cat 5 liver was weakly positive for *T. gondii* DNA. Lung sections from Cats 3 and 6 had previously been tested using the nPCR *dhps* around the time of death for molecular confirmation of the post-mortem diagnosis and were PCR positive. DNA from all cat tissues was also tested for *T. gondii* DNA by nPCR B1 by which Cat 2 lung tissue and Cat 6 brain tissue were found to be positive. Partial genotypes were obtained using PCR-RFLP analyses for lung tissue from Cat 2 and brain tissue from Cat 6 (see Table 14). For Cat 2, RFLP analysis results were consistent with a Type II strain at the SAG1, (5' + 3') SAG2, and SAG3 loci, but with a Type I strain at the B1 locus. For Cat 6, genetic characterisation was successful at 6 of the 8 loci, revealing alleles from Type I (SAG3, L358), Type II (SAG2, Apico, GRA6), and Type X (B1). For Cat 2, due to the presence of Type II alleles at SAG1, 5' – 3' SAG2, and SAG3, possible known genotypes included ToxoDB #1, #3, #4, #39, #127, #128, #129, or #168. Comparison of the RFLP pattern of Cat 6 to those in the ToxoDB database (Gajria et al. 2007) showed no match with previously identified genotypes, suggesting the presence of a unique, Type X (ToxoDB #5)-like genotype in this animal. Further screening using the PCR apicomp identified protozoal DNA in the tissues of Cats 1, 2, 3, 5 and 6, which had previously shown immunopositive organisms by IHC. Sequencing was attempted and was successful for Cats 2, 3, and 5 (see Table 13); amplicons were found to have 99 % nucleotide similarity to *T. gondii* reference sequences for the ITS-1 gene (GenBank Accession Number KP895866.1; Chemoh et al., 2016). Sequencing was not attempted for Cats 1 and 6 due to insufficient amplification.

5.3.5 Transmission electron microscopy

Transmission election microscopy was possible for Cats 1, 2, 4, and 5, but tissue quality for Cats 3 and 6 was poor. Ultrastructurally, small clusters of *T. gondii* organisms were identified in the stomach of Cat 1 (Fig 12A – D). These organisms had characteristic apical structures including conoid, rhoptry, and micronemes, consistent with *T. gondii* tachyzoites (Dubey et al., 1998; Speer et al., 1999; Dubey, *pers. comm.,* 2019).

5.3.6 Final diagnosis

Overall, 5/6 of the cats examined (Cats 1, 2, 3, 5, and 6) were given a definitive diagnosis of toxoplasmosis (see Table 13), according to the criteria outlined in the methods. Cat 4 was identified in the database as a suspected case of toxoplasmosis, and histological examination showed a single cluster of structures consistent with protozoal infection within the brain. Brain sections, however, were negative on IHC, and while brain was weakly positive for *T. gondii* DNA by PCR, this result could not be confirmed by PCR-RFLP genotyping or sequencing. Cat 4, therefore, did not satisfy the criteria for a definitive diagnosis of toxoplasmosis.

5.4 Discussion

One of the aims of this study was to describe feline cases of toxoplasmosis in New Zealand, as they had not previously been reported on in detail in the literature. Between 1991 – 2017, nine cases in the Pathobiology Group database at the School of Veterinary Science, Massey University, had a diagnosis of toxoplasmosis, based on evaluation of gross lesions and H&E stained tissue sections. Six of these cases had FFPE tissues available for further investigation. To describe the cases in detail, clinical histories, original pathology reports, and histological tissue sections were re-examined in this study, and IHC, PCR, and TEM carried out for confirmatory purposes. From the study results, it was possible to confirm *T. gondii* infection in five out of the six cats. In the remaining cat (Cat 4), focal cerebral toxoplasmosis

was suspected on the basis of protozoal-like structures on histology, but *T. gondii* could not be confirmed by either IHC or molecular methods.

Clinical toxoplasmosis is relatively uncommon in cats, but some cases have been described in the literature. The current study found that clinical signs, gross lesions and histological lesions in New Zealand cats with a definitive diagnosis of toxoplasmosis were generally in agreement with previous studies from overseas. Clinical signs included anorexia, dyspnoea, pyrexia and anisocoria, as reported previously (Hirth & Nielsen, 1969; Lappin et al., 1989; Dubey & Carpenter, 1993; Vollaire et al., 2005; Dubey & Prowell, 2013). Interstitial pneumonia, which appears to be the most common lesion in naturally-occurring fatal toxoplasmosis in cats (Hirth & Nielsen, 1969; Dubey & Carpenter, 1993; Jokelainen et al., 2012), was observed in each of the four cats in this study that had toxoplasmosis confirmed as the cause of death. *Toxoplasma* lesions were also present in the liver and brain in these animals. As summarised by Jokelainen et al. (2012), active toxoplasmosis should be suspected if a cat has acute interstitial pneumonia, acute necrotising hepatitis, or nonsuppurative meningoencephalitis. Brain lesions were also observed in the case of Cat 4, consisting of a single focus of gliosis, and one small cluster of tissue cysts with no associated inflammation. It is possible that Cat 4 had a latent *T. gondii* infection, as described by Montoya (2002) and Brennan et al. (2016), defined as the presence of tissue cysts without associated inflammation, and an absence of free tachyzoites. Although *T. gondii* could not be confirmed either by IHC or molecular methods in this case, the tissue cysts observed were likely to be *T. gondii*. Brain tissue was weakly positive for *T. gondii* DNA using the nPCR *dhps*, and this assay does not cross-react with other closely related protozoans, specifically *Neospora caninum* and *Sarcocystis* spp. (Chapter 7, Coupe et al., 2019). Furthermore, in latent *T. gondii* infections a low burden of bradyzoite cysts is expected (VanWormer et al., 2014), lowering the chance of parasite detection. Combined with repeat tissue sectioning necessary for conducting multiple tests, this could explain why only a single weak positive was obtained after several replicates using the nPCR *dhps* and why other tests were negative.

Of particular interest in this study was the case of Cat 1 because this animal had gastric lesions which appeared to be caused by *T. gondii*. Whilst intestinal involvement has been reported in 41.5 % fatal cases of generalised feline toxoplasmosis (*n* = 65) examined histologically (Dubey & Carpenter, 1993), gastric involvement in naturally occurring toxoplasmosis is rarely reported (Petrak & Carpenter, 1965; Ganji et al., 2003; Merzianu et al., 2005; McConnell et al., 2007). Gastric involvement seems only to have been reported on in detail in one previous naturally occurring feline case (McConnell et al., 2007) in which eosinophilic fibrosing gastritis and toxoplasmosis was diagnosed through histology and IHC. In that case, the pylorus was enlarged with gross, irregular thickening of the gastric wall. Biopsy of the gastric wall showed disruption of the muscularis by bands of fibrovascular tissue infiltrated with large numbers of eosinophils and smaller numbers of lymphocytes, plasma cells and histiocytes. Immunostaining showed the presence of multiple free *T. gondii* tachyzoites within inflammatory lesions. Gastric involvement has been documented in experimentally infected kittens that were given large doses of corticosteroids (Dubey & Frenkel, 1974). Smooth muscle lesions consisting of focal areas of myositis, sometimes associated with large numbers of *Toxoplasma* organisms, were seen in the stomachs of six out of 24 cats, and in the intestine of eight cats. Tachyzoites sometimes occurred along the blood vessels, and in one cat they were identified in the capillaries. Lesions observed in Cat 1 in the current study were similar to those in both previous reports. The stomach wall of Cat 1 was severely thickened, with pyloric infarction, and prominent granulation tissue and mixed cell inflammatory infiltrate in the adjacent stomach wall. Large numbers of *Toxoplasma*-like organisms were also seen throughout the stomach biopsy; free organisms observed were confirmed by IHC and TEM to be *T. gondii* tachyzoites and this cat was therefore given a definitive diagnosis of toxoplasmosis. It is possible that this cat had disseminated toxoplasmosis with gastric involvement as toxoplasmic gastritis appears to occur in the context of generalised disease (Dubey & Carpenter, 1993; Ganji et al., 2003; McConnell et al., 2007) and is very rare as an isolated lesion (Merzianu et al., 2005). Furthermore, clinical gastrointestinal signs as seen in the case of Cat 1 have been reported in up to 35% of cases of generalised disease (Petrak & Carpenter, 1965). It is not known if there was intestinal or other organ involvement in Cat 1, as this cat was lost to follow-up.

Infection with *T. gondii* is widespread in cat populations; the study presented in Chapter 3 showed that 61 % of domestic cats sampled in New Zealand were seropositive for anti-*T. gondii* antibodies. Despite widespread exposure, in most healthy cats, as in mice and humans, tachyzoite proliferation is thought to be contained by a robust innate immune response and the infection remains subclinical (for review see Yarovinsky (2014)), meaning that clinical toxoplasmosis cases are relatively rare (Henriksen et al., 1994; Jokelainen et al., 2012; Nagel et al., 2013; Brennan et al., 2016). Of particular interest in this study was whether variant Type II *T. gondii* was associated with clinical feline toxoplasmosis cases because in New Zealand this strain may be particularly pathogenic, having been identified as the cause of fatal disseminated toxoplasmosis in Hector's dolphins (Roe et al., 2013) and native bird species (Howe et al., 2014), as well as systemic toxoplasmosis in a New Zealand fur seal (Roe et al., 2017). Partial genotypes were obtained for two out of five cats from which *T. gondii* was molecularly identified, that had both died as a result of active disseminated toxoplasmosis. What appeared to be a unique, atypical, Type X-like genotype was identified in one cat, with a Type II allele at the Apico locus. In the other cat, available RFLP results were consistent with a Type II-like strain with a type I allele at the B1 locus, however too few loci were amplified to confirm whether this was a known or unique genotype (Herrmann et al., 2010). Findings of this study cannot rule out the possibility that variant Type II is also present in New Zealand cats, as its absence may reflect the small sample size of the study as well as difficulties faced in amplifying the typing loci (see below). As discussed in Chapter 4 this strain has previously been found in cats overseas (Al-Kappany et al., 2010; Herrmann et al., 2010; VanWormer et al., 2014). For example, variant Type II was the most common strain identified from oocyst isolates (79 %, *n* = 68; Herrmann et al., 2010) in a large study of German owned domestic cats. This strain was also recently identified in seven owned domestic cats from Australia, four that died or were euthanised due to severe active toxoplasmosis, and three with asymptomatic latent infection (Brennan et al., 2016), demonstrating that this genotype can infect felids, but can be present without causing active disease. Further research is therefore needed in New Zealand to clarify whether variant Type II is a particular problem for certain species of endemic New Zealand wildlife, notably the Hector's dolphin, rather than all New Zealand animals.

The infecting genotype in clinical feline toxoplasmosis cases has only been determined in four previous studies (Spycher et al., 2011; Jokelainen et al., 2012; Dubey & Prowell, 2013; Brennan et al., 2016). Whilst variant Type II was detected in the four cats with active toxoplasmosis from Australia (Brennan et al., 2016) and in one case reported by Spycher et al. (2011) from Switzerland, other causal genotypes include clonal Type 12 (ToxoDB genotype #4) in one cat from the United States and clonal Type II (ToxoDB genotype #1) in six cats from Europe (Dubey & Prowell, 2013; Jokelainen et al., 2012). Results from these studies led Brennan et al. (2016) to conclude that genotypes associated with clinical feline toxoplasmosis are those endemic to the study area, as they were also found in healthy cats and other non-feline species. If this is the case, unique atypical genotypes may be prevalent in New Zealand alongside variant Type II, as observed in other countries in the Southern hemisphere (Lehmann et al., 2006; Dardé et al., 2013; Shwab et al., 2014; Mirza et al., 2017; Shwab et al., 2018).

As far as the author is aware, this is the first study to report an atypical Type X-like strain outside of the USA, based on PCR-RFLP results including the B1 locus. The B1 locus is not traditionally included in PCR-RFLP genotyping (Su et al., 2010, 2012), but has been adopted in some studies due to increased diagnostic sensitivity (as the B1 gene is multi-copy), and because it can also be used for strain typing via RFLP analysis (Grigg & Boothroyd, 2001; Miller et al., 2008; VanWormer et al., 2014; Shapiro et al., 2015; Brennan et al., 2016). RFLP cleaving patterns distinguish between clonal types I, II/III, and X, as well as atypical alleles (Shapiro et al., 2015). Atypical Type X *T. gondii* (ToxoDB #5) is one of the dominant strains of *T. gondii* in North America (Shwab et al., 2014), particularly in wildlife, and whilst it has been identified in humans (Howe & Sibley, 1995; Khan et al., 2011) as well as owned and free-ranging domestic cats, it has predominantly been found in the Southern sea otter (Miller et al., 2008; VanWormer et al., 2014; Jiang et al., 2018; Shapiro et al., 2019), for which it appears to be highly pathogenic (Shapiro et al., 2019).

Other proposed determinants of clinical toxoplasmosis and disease severity include cat age, concurrent infections, and immunosuppression, although severe toxoplasmosis has been documented in otherwise

healthy adult cats (Dubey & Carpenter, 1993; Dubey et al., 2009; Spycher et al 2011; Jokelainen et al., 2012; Nagel et al., 2013; Brennan et al., 2016). In this study, no clear association between host-related factors and acute toxoplasmosis was observed. The literature advises that congenital infections in kittens are more likely to result in fatal disseminated toxoplasmosis (Dubey et al., 2009). Of the four cats diagnosed with fatal disseminated toxoplasmosis here, two were eight-week old kittens (Cats 3 and 6), and it is possible that these infections were due to transplacental transmission of *T. gondii*, as at eight weeks of age these animals are unlikely to have been exposed to *T. gondii* through consumption of infected prey. Findings suggest, however, that age alone was not a determinant of disease in the study cats as two of the four disseminated cases were in adults (Cats 2 and 5) and Cat 1, which had toxoplasmic gastritis, was also an adult. Whether the study animals were positive for immunosuppressive diseases such as feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV) is unknown. There appears to be a low prevalence of FeLV in New Zealand (Gates et al., 2017; Luckman & Gates, 2017) but New Zealand has one of the highest rates of FIV infection in the world (Gates et al., 2017; Luckman & Gates, 2017; Stickney et al., 2020). No test results for either FIV or FeLV were included in the case histories for the cats in this study. Three of the six cats in the study (Cats 1, 2, and 4) had been administered potentially immunosuppressive drugs, although the dosage was not reported, and it is therefore possible that drug-induced immunosuppression may have played a role in disease in these cases. It is also possible that host genetic factors such as major histocompatibility complex (MHC) haplotype and specific genetic polymorphisms play an important role in the susceptibility to and severity of toxoplasmosis in cats, as suspected in humans and other species (McLeod et al., 1993; Mack et al., 1999; Jamieson et al., 2008; Roe et al., 2013; Brennan et al., 2016); evaluation of this possibility was beyond the scope of this study.

This study also highlighted issues surrounding confirming a diagnosis of toxoplasmosis in cats. Immunohistochemistry for *T. gondii* performed well and this method has been used alone for confirmation of diagnosis in postmortem investigations (e.g. Henriksen et al., 1994; McConnell et al., 2007; Brennan et al., 2016). However, in the current study, one cat (Cat 2) was immunopositive for both *N. caninum* and *T. gondii*. It is unlikely that Cat 2 had a dual infection, and the positive result was

considered more likely to be due to *N. caninum* antibody cross-reactivity with *T. gondii* parasites (Van Maanen et al., 2004; Gondim et al., 2017). This conclusion was reached because neosporosis has only been induced experimentally in cats thus far (Dubey et al., 1990) and because the presence of *T. gondii* DNA was confirmed in the tissue section by PCR-RFLP genotyping and sequencing of the apicomplexan ITS-1 gene. As no immunoreaction to anti-*N. caninum* antibody was observed in the other cases, however, further tests were necessary to rule out the possibility of concurrent neosporosis.

Since the advent of PCR, molecular assays are often used in combination with histology and/or immunohistochemistry, for confirmatory purposes and to determine the genotype of infecting *T. gondii* (e.g. Spycher et al., 2011; Jokelainen et al., 2012; Nagel et al., 2013; Brennan et al., 2016). Although PCR and/or direct sequencing confirmed the presence of *T. gondii* DNA in five of the six study cats, it was found that PCR on FFPE tissues can be negative despite an abundance of immunopositive organisms on IHC, and that results were not consistent between replicates, particularly for the nPCR *dhps*. The most reliable PCR assay, in terms of agreement with H&E observations and IHC results, appeared to be the conventional PCR targeting the apicomplexan ITS-1 gene, combined with direct sequencing, in line with results from a recent study of *T. gondii* oocyst shedding in cats (Chemoh et al., 2016). Possible reasons for this, assuming *T. gondii* DNA was present in all IHC positive tissues, include the number of gene copies of the PCR target amplified, and effects of formalin fixation and long-term storage of FFPE samples on DNA quality. There are an estimated 110 copies of the ITS-1 marker in the *T. gondii* genome but approximately 35 copies of the B1 gene , whilst the *dhps* gene is likely to be single-copy (as are the PCR-RFLP genotyping loci used in this study) (Su et al., 2010), meaning the probability of detection of the ITS-1 gene may be higher. Compounding this issue, obtaining nucleic acids from FFPE specimens can be challenging, especially after storage for several years. The quality of DNA in terms of amplifiable length may be significantly reduced and PCR inhibition may occur (Dietrich et al., 2013; Ademà et al., 2014; Guyard et al., 2017; Watanabe et al., 2017). Likewise, extended formalin fixation times are known to adversely affect downstream molecular analyses (Ferrer et al., 2007; Chapter 2). Whilst all tissues in this study were processed following a maximum of one week in formalin, the age of the FFPE specimens ranged from 6 – 20 years. It is therefore possible that age-

related DNA fragmentation reduced the amplifiable length of a significant proportion of *T. gondii* DNA below the size of the PCR markers used in the study (*dhps* = 450 bp; B1 = 530 bp; ITS-1 = 550 bp).

Transmission electron microscopy was employed as an additional means of identifying *T. gondii* organisms in infected tissues (Parker et al., 1981; Ploeg et al., 2018). There are also problems to consider with the use of TEM. Processing of FFPE tissue for TEM can give useful results but is not ideal as artifacts may be prominent (Cheville & Stasko, 2014). This study found that there was poor tissue preservation after retrieval for two of the six cats. It was therefore difficult to get sufficient visual clarity needed to identify *T. gondii* parasites, and to distinguish between *T. gondii* organisms and other protozoan parasites with similar ultrastructures, particularly *Neospora* spp. and *Hammondia* spp. (Speer et al., 1999; Dubey & Sreekumar, 2003), although *Hammondia* spp. is not believed to cause lesions in cats (Dubey & Sreekumar, 2003), and neosporosis has only been documented after experimental infection (Dubey et al., 1990). Differentiation between *T. gondii* bradyzoites and tachyzoites, following Dubey et al. (1998), was also challenging due to sub-optimal tissue preservation, but free tachyzoites in the stomach of in one cat (Cat 1) were successfully visualised (Dubey, *pers comm*., 2019).

This study was successful in providing detailed descriptions of clinical signs, gross, and histopathological lesions of clinical toxoplasmosis in five domestic cats from New Zealand. Of note, a case of toxoplasmic gastritis was documented. This is a rare manifestation of toxoplasmosis only previously reported in one other naturally infected cat overseas. The immune status of the study cats was not known, so it was not possible to ascertain whether clinical toxoplasmosis occurred in the context of immunosuppression in these animals. A definitive diagnosis of toxoplasmosis was given with confidence to five out six cats investigated but confirmation of the diagnosis using IHC, PCR, and TEM, proved to be somewhat challenging, highlighting a need to consider the best means of reaching a definitive diagnosis for subsequent cases. In light of the results of this study, the following recommendations can be made, taking into account that confirmatory testing must be practical, cost-effective, and specific for the

disease: 1. the specificity of the IHC for *T. gondii* should be thoroughly assessed to rule out cross-reactivity and non-specific antibody binding; 2. the validated IHC test should be used in conjunction with the apicomplexan PCR combined with sequencing. Where resources permit, PCR testing in triplicate is advised; 3. DNA extraction from FFPE tissues should be carried out shortly after the samples have been processed to ensure DNA quality is optimal for molecular analysis or, when possible, frozen samples should be kept.

The study also aimed to determine the genotype of *T. gondii* in the clinical cases, as variant Type II *T. gondii* is hypothesised to be widespread in New Zealand or to be particularly virulent, being the predominant genotype identified so far in endemic wildlife cases of toxoplasmosis. No evidence of this genotype was confirmed in the study animals, but further research is needed due to the small sample size and difficulties faced with PCR amplification. Although the presence of variant Type II *T. gondii* cannot be ruled out, it appears that unique atypical genotypes also occur in New Zealand and can cause disease in local domestic cats. Results support the hypothesis that variant Type II *T. gondii* is especially pathogenic for certain endemic wildlife species.

Table 12 Summary of clinical histories, gross, cytological, and histological lesions for six cats diagnosed with toxoplasmosis by the Pathobiology Group at the School of Veterinary Science, Massey University, New Zealand. Cases and histological tissue sections were re-examined by an individual veterinary pathologist for consistency

Cat ID	Date	Sex	Age	Submission Type	Diagnosis	Presenting signs and clinical findings	Gross lesions	Histological lesions consistent with toxoplasmosis	Use of immunosuppressive drugs
1 28792	30/01/98	F	12 y	Biopsy	toxoplasmic gastritis	A, V, WL	Stomach	Stomach	Y
2 **39525**	24/11/06	M	Adult	Post-mortem	disseminated toxoplasmosis	R anti-*T. gondii* IgG titre 1:1024 Severe interstitial pulmonary nodules Sunken eyes Pale buccal and conjunctival mucous membranes	Liver Lungs	Liver Lungs Heart Eye Pancreas Spleen Kidney	Y

Cat ID	Date	Sex	Age	Submission Type	Diagnosis	Presenting signs and clinical findings	Gross lesions	Histological lesions consistent with toxoplasmosis	Use of immunosuppressive drugs
3 **40038**	20/04/07		8 w	Post-mortem	disseminated toxoplasmosis	SD, V, C Poor body condition Pallor	Liver Lungs Brain	Liver Lungs Brain Heart Kidney	N
4 **44301**	04/12/09	M	10 y	Post-mortem	cerebral toxoplasmosis (focal)	WL, D, A, IBD, AZ, HA, AN, HN, USG 1.020, N, ANC, DP, O Thin body condition Jaundice	Liver Heart Parathyroids Kidneys	Liver Lungs Brain Eye Pancreas Kidney Intestine Thyroid	Y

Cat ID	Date	Sex	Age	Submission Type	Diagnosis	Presenting signs and clinical findings	Gross lesions	Histological lesions consistent with toxoplasmosis	Use of immunosuppressive drugs
5 **45286**	18/08/10	M	13 y	Post-mortem	disseminated toxoplasmosis	A, P, V, D, CL, DP, ANC Good body condition	Liver Lungs Pancreas	Liver Lungs Brain Eye Pancreas Bladder	N
6 **47035**	03/01/12	F	8 w	Post-mortem	disseminated toxoplasmosis	No history given Moderate body condition		Liver Lungs Brain	N

A: anorexia; AN: anaemia; ANC: anisocoria; AZ: azotaemia; C: cyanosis; CL: cholestatic liver disease; D: diarrhoea; DP: dyspnoea; HA: hyopalbuminaemia; HN: hypernatraemia; IBD: inflammatory bowel disease; N: neurologic disease; O: obtundation; P: pyrexia; R: respiratory disease; SD: sudden death; USG: urine specific gravity; V: vomiting; WL: weight loss

Table 13 Summary of histological, immunohistochemical, transmission electron microscopic (TEM), and molecular observations from six cats diagnosed with toxoplasmosis by the Pathobiology Group at the School of Veterinary Science, Massey University, New Zealand. Findings were used to obtain a final interpretation of the cases and reach a definitive diagnosis of toxoplasmosis or otherwise.

Cat	**Tissue**	**H&E** *T. gondii* **organisms**	**IHC** *T. gondii*	**IHC** *N. caninum*	**PCR** *T. gondii* **DNA** *dhps* **gene**	**PCR** *T. gondii* **DNA** **B1 gene**	**PCR** **apicomplexan DNA (sequence confirmed)**	**TEM**	**Final Interpretation**
1 **28792**	stomach	+	+	-	- -/- -/- -	-	+[a]	pos	toxoplasmosis
	lymph node	-	+	nd	-	-	-	nd	
2 **39525**	lung	+	+	+[a]	- +/- -/- -	+	+ (y)	neg	toxoplasmosis
	liver	-	+	nd	-	-	-	nd	
	heart	-	+	nd	-	-	-	nd	
	pancreas	-	+	nd	-	-	-	nd	
	eye	-	?[e]	nd	-	-	-	nd	

Cat	Tissue	H&E *T. gondii* organisms	IHC *T. gondii*	IHC *N. caninum*	PCR *T. gondii* DNA *dhps* gene	PCR *T. gondii* DNA B1 gene	PCR apicomplexan DNA (sequence confirmed)	TEM	Final Interpretation
3 **40038**	brain	+	+	-	- -/- -/- -	-	+ (y)	?[h]	toxoplasmosis
	liver	?[b]	+	nd	-	-	+[a]	nd	
	lung	-	+	nd	+[c]/-	-	+[a]	nd	
	heart	+	+	nd	-	-	+[a]	nd	
	spleen	+	+	nd	-	-	+[a]	nd	

Cat	Tissue	H&E *T. gondii* organisms	IHC *T. gondii*	IHC *N. caninum*	PCR *T. gondii* DNA *dhps* gene	PCR *T. gondii* DNA B1 gene	PCR apicomplexan DNA (sequence confirmed)	TEM	Final Interpretation
4 **44301**	brain	+	-[d]	-	- -/+[a] -/- -	-	-	neg	suspect
	lung	-	-	nd	-	-	-	nd	
	liver	-	-	nd	-	-	-	nd	
	eye	-	?[e]	nd	-	-	-	nd	

Cat	Tissue	H&E *T. gondii* organisms	IHC *T. gondii*	IHC *N. caninum*	PCR *T. gondii* DNA *dhps* gene	PCR *T. gondii* DNA B1 gene	PCR apicomplexan DNA (sequence confirmed)	TEM	Final Interpretation
5 **45286**	lung	+	+	-	- -/- -/- -	-	-	neg	toxoplasmosis
	eye	+	?[e]	nd	-	-	-	nd	
	liver	+	+	nd	+[a]	-	+ (y)	nd	
	bladder	+	+	nd	-	-	+[a]	nd	
	brain	+	+	nd	-	-	+[a]	nd	
	pancreas	+	nd[g]	nd	nd[g]	nd[g]	nd[g]	nd	

Cat	Tissue	H&E *T. gondii* organisms	IHC *T. gondii*	IHC *N. caninum*	PCR *T. gondii* DNA *dhps* gene	PCR *T. gondii* DNA B1 gene	PCR apicomplexan DNA (sequence confirmed)	TEM	Final Interpretation
6 **47035**[f]	brain	+	+	-	- -/- -/- -	+	+[a]	nd	toxoplasmosis
	liver	?[b]	+	nd	-	-	-	?[h, i]	
	lung	?[b]	+	nd	+[c]/-	-	-	nd	

For PCR results, "+" indicates presence of a band (positive), "-" indicates absence of a band (negative), and each DNA extraction is separated by a "/"; [a] weak positive; [b] possible presence of *T. gondii* organisms; [c] PCR result already available – two cases were tested around time of death for molecular confirmation of the post-mortem diagnosis; [d] region with cysts on H&E was absent when re-sectioned for IHC; [e] melanin granules obscured interpretation, [f] case was presented frozen, all tissues displayed a combination of autolysis and freeze-thaw artefact; [g] FFPE tissue unavailable for further analysis; [h] poor preservation of tissue after processing for TEM;[i] Insufficient brain tissue remained for TEM reprocessing so liver tissue was used instead; nd = not done

Table 14 Summary of multilocus PCR-RFLP typing for *Toxoplasma gondii* reference strains and for tissues obtained from New Zealand cats that were diagnosed with fatal toxoplasmosis

***Toxoplasma gondii* isolate/strain**	**SAG1[a]**	**(5' + 3') SAG2[a]**	**SAG3[a]**	**L358[a]**	**PK1[a]**	**GRA6[a]**	**Apico[a]**	**B1[b]**	**Genotype**
RH88 (Type I)	I	I	I	I	I	I	I	I	ToxoDB #10
PTG (Type II)	II or III	II	II	II	II	II	II	II or III	ToxoDB #1
Variant Type II	II or III	II	II	II	II	II	I	II or III	ToxoDB #3
CTG (Type III)	II or III	III	III	III	III	III	III	II or III	ToxoDB #2
Type 12 (Type X)	u-1	II	II	I	II	II	I	X	ToxoDB #5
Cat 2	II or III	II	II	-[c]	-[c]	-[c]	-[c]	I	
Cat 6	-[c]	II	I	I	-	II	II	X	

[a] PCR-RFLP following Su et al. (2010) [b] PCR-RFLP following Shapiro et al. (2015) [c] PCR was performed but *T. gondii* DNA was not amplified.

Figure 9 Histological lesions. [A] Section of stomach from Cat 1 showing severe pyloric infarction, with prominent granulation tissue and mixed cell inflammatory infiltrate in the adjacent stomach wall. Large numbers of small, mainly spherical, organisms seen, often in clusters of one hundred (arrows) or smaller aggregates containing 2 – 6 up to 10 – 20 organisms, considered to be *Toxoplasma gondii* pseudocysts containing tachyzoites (Dubey, *pers. comm.*, 2019). [B], [C], and [D] Photomicrographs from the stomach biopsy of Cat 1 showing extensive parasitism with *Toxoplasma gondii* immunopositive pseudocysts (arrows) and free tachyzoites (arrowheads) (Dubey, *pers. comm.*, 2019). Original magnifications ×40 [A], ×20 [B], ×40 [C], ×4 [D].

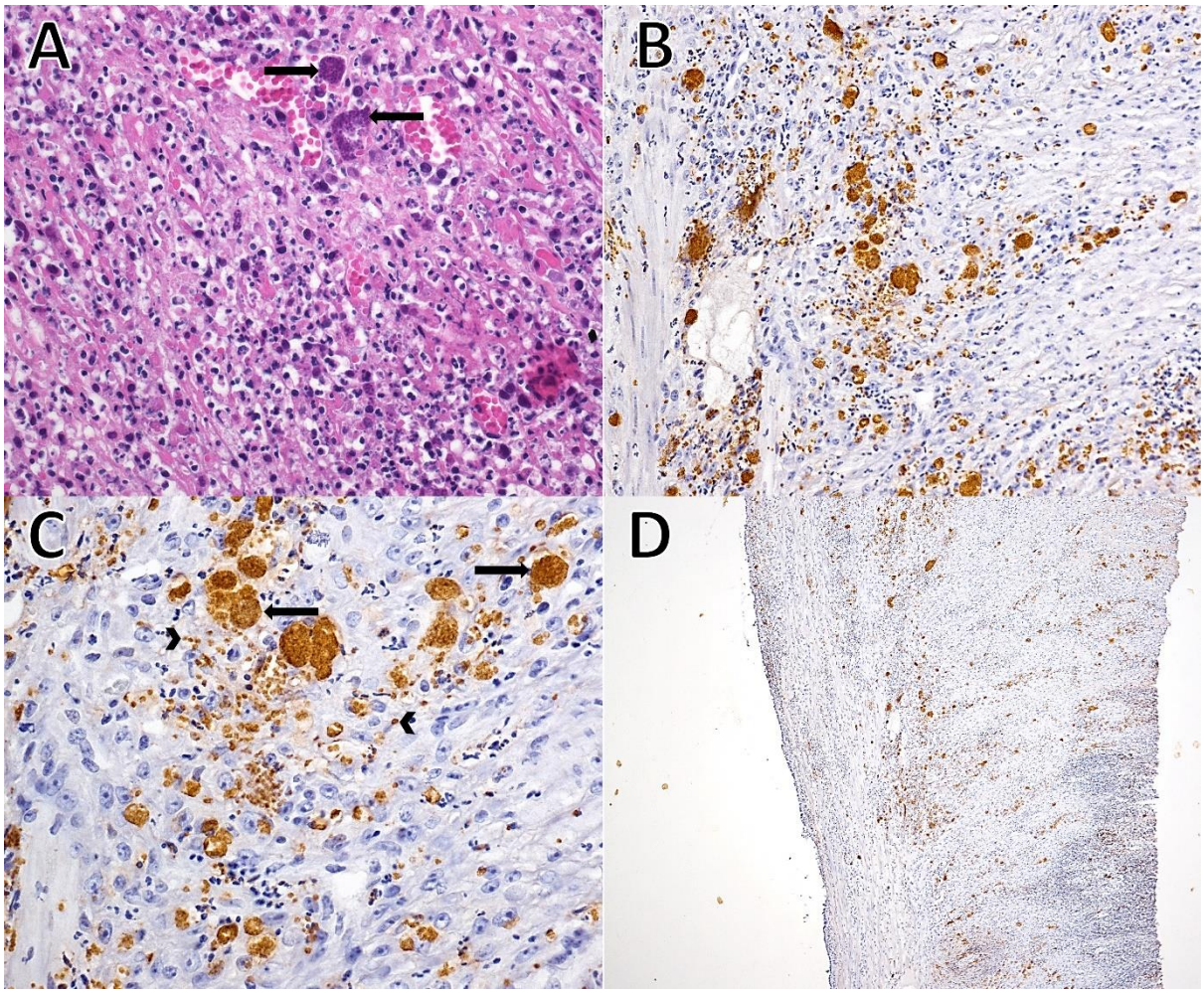


Figure 10 Histological lesions. [A] Section of brain from Cat 3 showing moderate gliosis, with occasional *Toxoplasma gondii* tissue cysts (arrow). [B], [C] The same section of brain immunostained using anti-*T. gondii* antibody. Photomicrographs show occasional *T. gondii* immunopositive tissue cysts (arrows) and numerous tachyzoites (arrowheads). Original magnifications ×20 [A] and [B], ×40 [C].

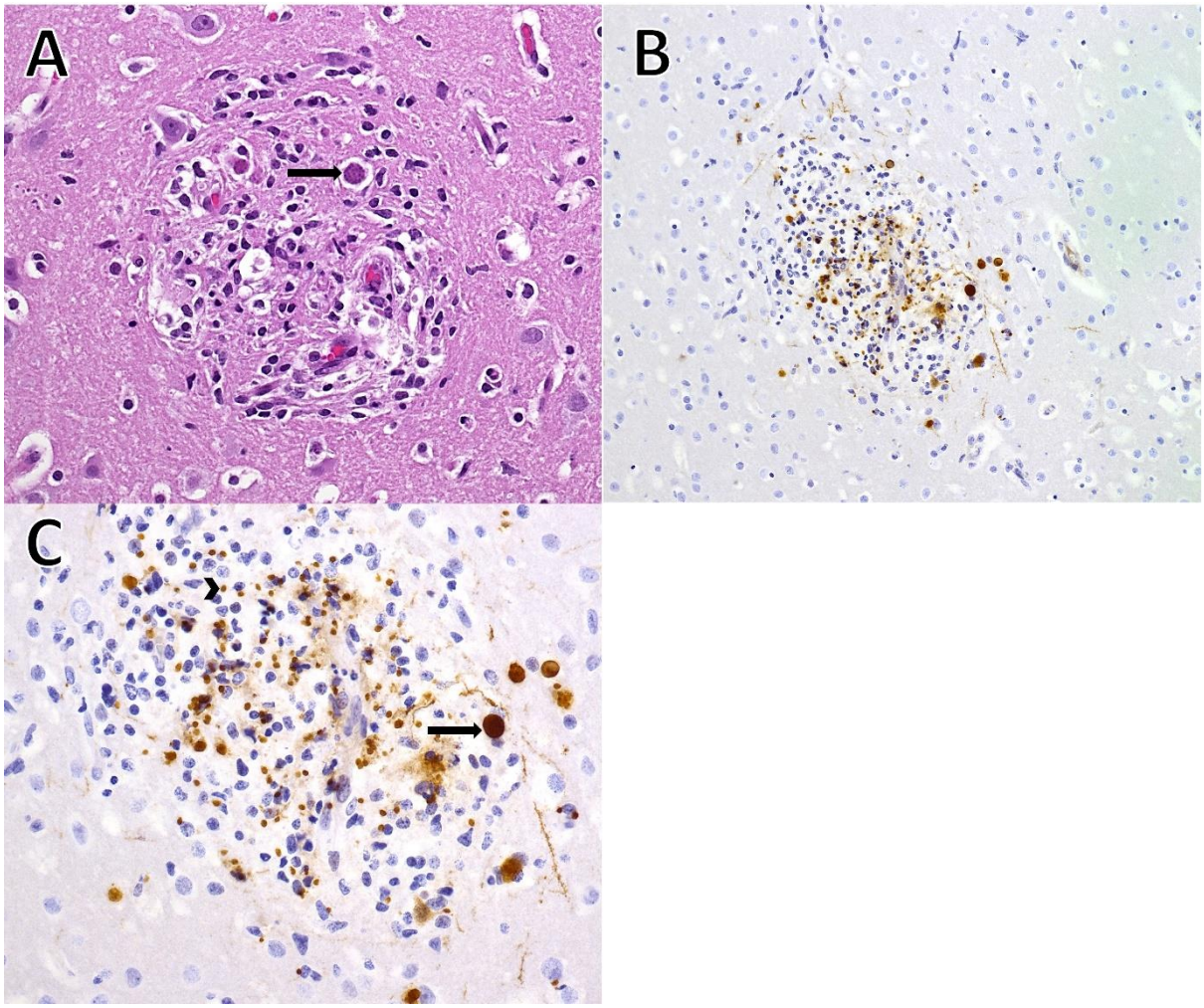


Figure 11 Histological lesions. [A] and [B] Section of lung from Cat 2 showing positive reaction of the tissue to anti-*Neospora caninum* antibody. [C] and [D] Photomicrographs of the same section of lung showing stronger and more extensive reaction of the tissue to anti-*Toxoplasma gondii* antibody, with immunostained *T. gondii* tachyzoites (arrowheads) and tissue cysts (arrows) observed. No other cats included in this study had tissues showing cross-reactivity with anti-*Neospora caninum* antibody by immunohistochemical analysis. [E] Section of brain from Cat 6 showing multiple *T. gondii* tissue cysts (arrows) and a small number of tachyzoites (arrowheads) which were strongly immunopositive using anti-*T. gondii* antibody. [F] No immunostaining was evident on the same section of brain using anti-*N. caninum* antibody. Note that tissue cysts (arrows) are easily visible even without a positive reaction against the secondary antibody. Original magnifications ×20 [A], ×40 [B] and [C], ×60 [D], ×40 [E] and [F].

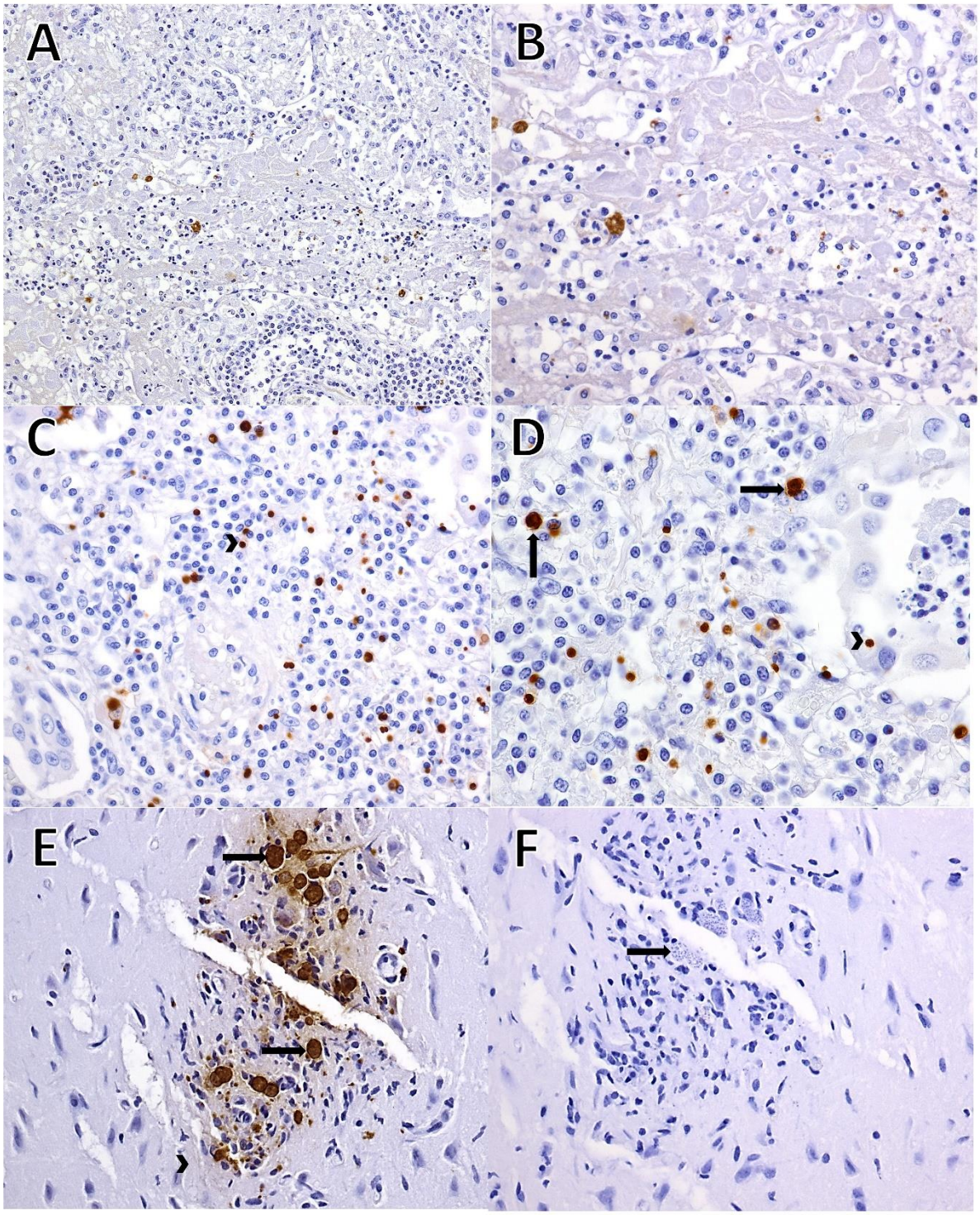
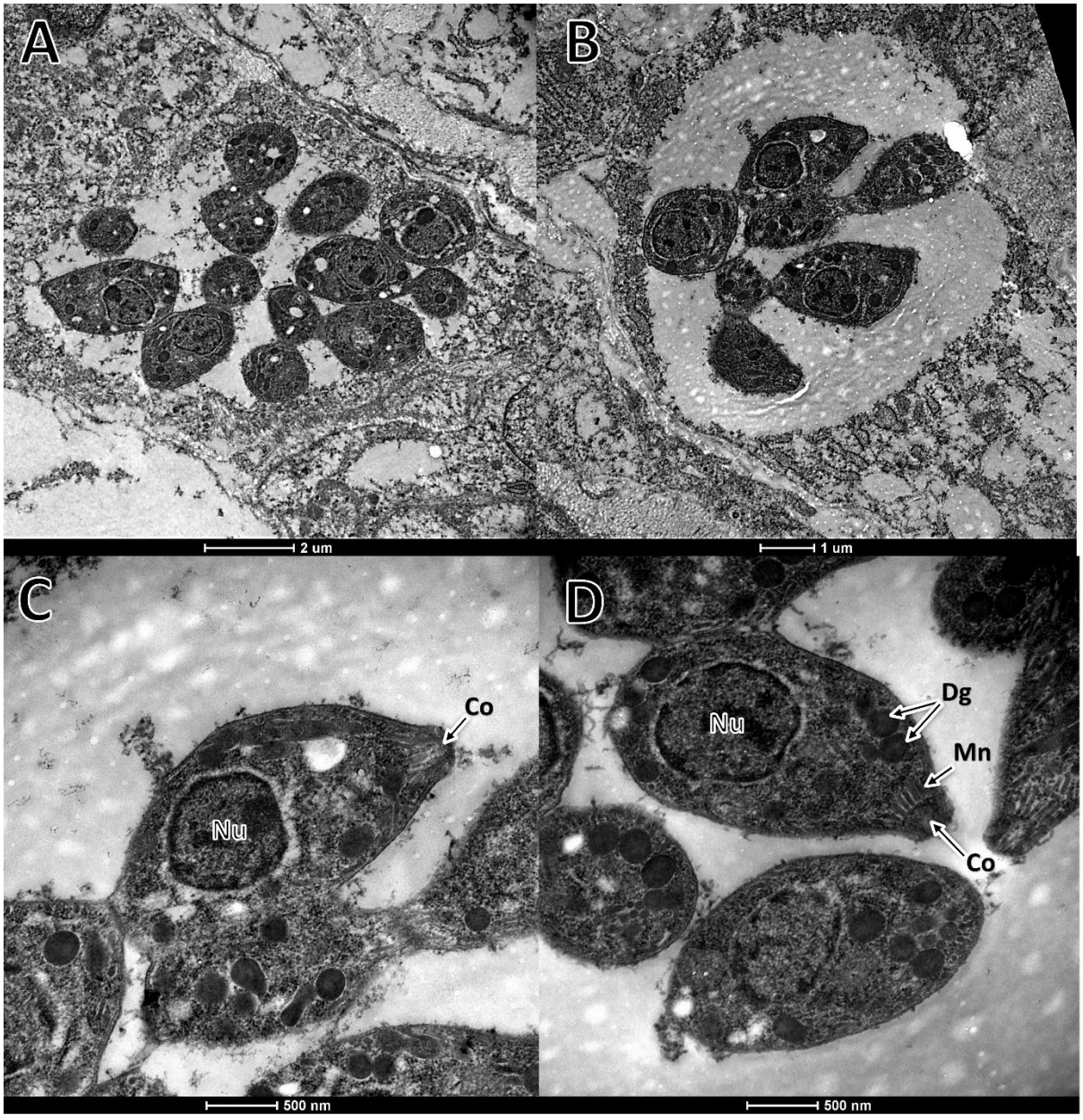

A
B
C
D
E
F

Figure 12 Transmission electron micrographs of free *Toxoplasma gondii* tachyzoites in the stomach of Cat 1. Nu = nucleus; Co = conoid; Mn = micronemes; Dg = electron-dense granules. Original magnifications ×8200 [A], ×9900 [B], ×26500 [C] and [D].



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6 First report of *Toxoplasma gondii* sporulated oocysts and *Giardia duodenalis* in commercial green-lipped mussels (*Perna canaliculus*) in New Zealand

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Coupe, A., Howe, L., Burrows, E., Sine, A., Pita, A., Velathanthiri, N., ... & Roe, W. D. (2018). First report of *Toxoplasma gondii* sporulated oocysts and *Giardia duodenalis* in commercial green-lipped mussels (*Perna canaliculus*) in New Zealand. *Parasitology research*, 117(5), 1453-1463 (Appendix 6i).

6.1 Introduction

Toxoplasma gondii, *Cryptosporidium* spp., and *Giardia* spp. are water-borne protozoan parasites of significant medical and veterinary importance. Found worldwide, (oo)cysts of these parasites are released into the environment, often in vast quantities. *Cryptosporidium* spp. and *Giardia duodenalis* (oo)cysts are shed in faeces of a variety of infected animals, including humans (Fayer et al., 1998; Thompson, 2004) but *T. gondii* oocysts are only excreted by cats, primarily after first infection (Dabritz & Conrad, 2010). Freshwater sources contaminated with *Cryptosporidium hominis*, *C. parvum* or *Giardia duodenalis* (synonyms *G. intestinalis* and *G. lamblia*) (oo)cysts are well-associated with human outbreaks of diarrhoea, which can be recurrent and severe (Miller et al., 2005; Lucy et al., 2008; Hohweyer et al., 2013; Willis et al., 2013). Drinking water contaminated with *T. gondii* oocysts has been linked to outbreaks of acute toxoplasmosis in humans (Bowie et al., 1997; Jones & Dubey, 2010). Human infection with *T. gondii* can also be serious, particularly for pregnant women, congenitally-infected children, and immunosuppressed people, resulting in encephalitis, chorioretinitis, and abortion (Tenter et al., 2000; Sukthana, 2006). An estimated 30 % of the entire human population is chronically infected with *T. gondii* and latent infection has been linked to a number of psychological disorders (Tenter et al., 2000; Webster et al., 2013; Ene et al., 2016; but see Sugden et al., 2016).

Water-sampling studies show that highly resistant (oo)cysts of *Cryptosporidium* and *Giardia* spp. can pollute estuarine and marine ecosystems, likely transported in freshwater surface runoff (Johnson et al., 1995; Fayer, 2004; Betancourt et al., 2014). Whilst direct detection of *T. gondii* in seawater is more challenging (Karanis et al., 2013; Wells et al., 2015), this parasite is believed to contaminate the coastal environment in a comparable manner (Miller et al., 2002; Conrad et al., 2005; Dubey & Jones, 2008; VanWormer et al., 2013, 2016). A number of studies have shown that all three protozoans are present in estuarine and marine shellfish (Fayer et al., 1998; Miller et al., 2005a; Willis et al., 2013; Shapiro et al., 2015; Staggs et al., 2015). Bivalve shellfish such as mussels, oysters, clams, and cockles are filter feeders that concentrate suspended particles, including pathogens, from water. Due to this ability to remove and concentrate waterborne contaminants, bivalves are now recognised worldwide as potential bio-indicators of aquatic pollution (Graczyk et al., 1999; Miller et al., 2005b; Shapiro et al.,

2015) and may further represent a significant health risk to consumers (Downey & Graczyk, 2007; Gilbert et al., 2007; Robertson, 2007; Jones & Dubey, 2010; Smith & Nichols, 2010; Chiang et al., 2014).

In New Zealand there is very limited information on *T. gondii*, *Cryptosporidium* and *Giardia* spp. in the marine environment and presence of these parasites has not yet been investigated in shellfish (Gilbert et al., 2007). Previous studies have shown that *Cryptosporidium* and *Giardia* spp. are prevalent in freshwater sources (Brown et al., 1992; Ionas et al., 1998; Till et al., 2008) and consumption of recreationally-sourced shellfish has been linked to two cases of giardiasis in humans reported in the country (Scholes et al., 2009). Toxoplasmosis has recently been identified as a cause of mortality for the endangered Hector's dolphin (*Cephalorhyncus hectori*), found only in New Zealand coastal waters (Roe et al., 2013), but little data have been gathered on human toxoplasmosis in New Zealand. Shellfish consumption has, however, been identified as a significant risk factor for human toxoplasmosis in the United States (OR = 2.22, $p < 0.05$) (Jones et al. 2009) and Taiwan (OR = 3.7, $p = 0.008$) (Chiang et al., 2014). The green-lipped mussel (*Perna canaliculus*) is an endemic shellfish species that occurs naturally throughout coastal New Zealand and these mussels are commonly harvested recreationally for human consumption. In addition, *P. canaliculus* is farmed commercially in New Zealand, and it is estimated that over 63, 000 metric tons of green-lipped mussels are consumed annually by New Zealanders alone (King & Lake, 2013). In order to investigate possible links between human consumption of green-lipped mussels and protozoal infections, two molecular assays were validated for the detection of *T. gondii* DNA and RNA in mussel haemolymph. The validated tests and two additional molecular assays, each detecting a different gene target, were then used to investigate the prevalence of potentially zoonotic protozoans *Toxoplasma gondii*, *Cryptosporidium* spp. and *Giardia duodenalis,* in commercially- sourced green-lipped mussels.

6.2 Methods

6.2.1 Mussel sampling and processing

A total of 104 green-lipped mussels (*Perna canaliculus*) that had been commercially grown for human consumption in ocean farms were collected fresh from eight different commercial outlets in New Zealand at seven time points between September 2013 and November 2015 (see Table 15). It was not possible to obtain details on seller collection dates or sources. Mussels were kept cool and transported to Massey University, Palmerston North, New Zealand, where they were processed within 24 h of collection. Outer shell surfaces were washed by hand with tapwater before a notch was filed to allow aseptic extraction of haemolymph from the posterior adductor muscle using a sterile 22-gauge needle (Shapiro et al., 2015). Aspirated haemolymph was stored in sterile Eppendorf tubes at -20°C for DNA/RNA extraction and PCR analysis. Before processing, haemolymph was thawed and 100 µl haemolymph per mussel was centrifuged at 20,000 x g for 10 mins, the supernatant removed, and the haemocyte cell pellet suspended in 100 µl PBS (1X, pH 7.4).

6.2.2 DNA extraction

DNA was extracted from individual mussel samples using DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany), following procedure detailed in Shapiro et al. (2015). Extraction controls using PBS (1X, pH 7.4) were included. DNA was stored at -20°C until used for molecular analysis. Not all mussels had sufficient haemolymph remaining for DNA extraction and testing for presence of *Cryptosporidium* spp. and *Giardia duodenalis*.

6.2.3 Detection of *Cryptosporidium* spp. and *Giardia duodenalis* DNA by nested PCR

Nested and semi-nested PCR assays were used to amplify fragments of the 60kDa glycoprotein (GP60) gene of *C. hominis* and *C. parvum* (800 – 850 bp) and the glutamate dehydrogenase (*gdh*) gene found in all assemblages of *G. duodenalis* (432 bp) (Table 16). PCRs were performed as previously described (Garcia-R et al., 2017). All nPCR runs included positive and negative controls. Reactions were visualised

on ethidium bromide stained 1 % agarose gels. Positive amplicons were purified by ethanol precipitation and submitted to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequences were analysed using Geneious software R10 (Biomatters, Auckland, New Zealand) and compared with reference sequences for *Cryptosporidium* species and *G. duodenalis* assemblages using a BLAST search (GenBank database, NCBI).

6.2.4 Detection of *T. gondii* DNA by nested PCR

A spiking experiment was conducted in order to determine the sensitivity of the *T. gondii* nested PCR (nPCR) used in this study (Shapiro et al., 2015). Haemolymph, from green-lipped mussels previously determined to be negative for *T. gondii* DNA in duplicate runs of the nested PCR used in this study, was pooled and divided into 100 µl aliquots. Sporulated, heat-inactivated (80^{o}C for 20 min) *T. gondii* oocysts (Type II, M4 strain, originally obtained from Lee Innes of the Moredun Research Institute, Edinburgh, Scotland), were quantified and a ten-fold serial dilution prepared using sterile PBS (1X, pH 7.4). Dilutions were added to haemolymph aliquots at concentrations of 1000, 100, 50, 10, 5, and 1 oocyst(s) per 100 µl haemolymph. DNA was extracted for each oocyst concentration and the *T. gondii* nPCR assay run in triplicate per extraction.

A nPCR assay targeting a 450 bp fragment of the *T. gondii dhps* gene using FOOD1/2 and FOOD3/4 primers (Table 16) was employed to detect *T. gondii* DNA, as described in detail by Aspinall et al. (2002a), with the addition of 0.5 µl bovine serum albumin (BSA, 10 mg/ml) to each PCR reaction mix. A positive control from a known *T. gondii* isolate (incomplete strain S48, Toxovax®, MSD Animal Health, Wellington, New Zealand) (Hartley & Bridge, 1975), and a no template control (NTC) using ultrapure water were included in all nPCR runs. Assay specificity was determined by using DNA extracted from closely related protozoans, *Hammondia hammondi*, *Neospora caninum* and *Sarcocystis* spp. To confirm successful amplification, 10 µl of the final PCR product was run on a 1.5% agarose gel containing SYBR Safe (Thermo Fisher Scientific, Boston, MA, USA) and visualised with UV light using an E-Gel Imager (Life

Technologies, Carlsbad, CA, USA). Positive amplicons were purified using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA) and submitted to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems). Sequences were analysed using Geneious software 8.1.5 (Biomatters) and compared with reference sequences for *T. gondii* using a BLAST search (GenBank database, NCBI).

6.2.5 Genotyping *T. gondii* – nPCR-RFLP

The *T. gondii dhps* fragment amplified in the nPCR above is not suitable for genotyping as it is too highly conserved between strains (Aspinall et al., 2002a, b; Meneceur et al., 2008). Therefore nPCR-RFLP of the single-copy loci 3'SAG2, GRA6, and SAG1, and of the multi-copy B1 locus was attempted using previously described PCR protocols (Su et al., 2010; Shapiro et al., 2015).

6.2.6 RNA extraction

RNA was extracted from mussel haemolymph which tested positive for *T. gondii* DNA and which had sufficient haemolymph remaining. Extraction was carried out using RNeasy FFPE kits (Qiagen) following manufacturer's instructions with the following modifications. One hundred microliters of haemolymph was pelleted and resuspended in PBS, as described above, before addition of PKD Buffer (150 µl) and immersion in liquid nitrogen for 4 min/boiling water for 4 min. RNA was eluted in 25 µl RNase-free water and stored at -80°C until used for molecular analysis. Total RNA yield and purity was reviewed using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific).

6.2.7 Detection of *T. gondii* SporoSAG mRNA by RT-PCR

The analytical sensitivity of the *T. gondii* reverse-transcriptase PCR (RT-PCR) employed in this study was determined using RNA extracted from the serial dilution of sporulated *T. gondii* oocysts in PBS (see above). RNA was extracted for each oocyst concentration and the *T. gondii* RT-PCR assay was run in triplicate per extraction. RNA and DNA from 1000 tachyzoites (Type I S48 strain from the commercial

Toxovax® vaccine, Schering-Plough Animal Health, Wellington, New Zealand; Hartley & Bridge, 1975), as well as DNA from 1000 oocysts, were included as controls.

A conventional RT-PCR assay targeting a 71 bp fragment of the SporoSAG gene was utilised to amplify sporozoite-specific *T. gondii* mRNA using the primers SporoSAG F 5'-CGG ACA AAT GTG GCG TAC AC-3' and SporoSAG R 5'-GTG ATC TTG CGC CGA ACA C-3' (Travaillé et al., 2016) (Table 2). RT-PCR was performed using the SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen). Each 25 µl reaction mixture was prepared on ice and contained 2X Reaction Mix (12.5 ul), SuperScript® III RT/Platinum® *Taq* Mix (1 µl), 10 uM SporoSAG F (0.5 µl), 10 uM SporoSAG R (0.5 µl), RNA (5 µl; 30 – 50 ng RNA per reaction), and RNase-free water. Controls included a water blank and total RNA extracted from 1000 sporulated, heat-inactivated (80°C for 20 min), *T. gondii* oocysts. Cycling parameters were as follows: 55°C for 30 min (1 cycle); 94°C for 2min (1 cycle); 94°C for 15 sec, 60°C for 30 sec, 68°C for 45 sec (45 cycles); 68°C for 5 min (1 cycle) (Appendix 6ii). To confirm successful amplification, 10 µl of the final PCR product was run on a 2.5 % agarose gel stained with SYBR Safe (Thermo Fisher Scientific), before visualisation, as described above. The size of PCR amplicons was estimated by comparison with the O'RangeRuler 20 bp – 300 bp DNA Ladder (Thermo Fisher Scientific). A positive RT-PCR amplicon sample from 1000 sporulated oocysts was purified (PureLink PCR purification kit, Invitrogen) and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc) to confirm genomic sequence. The electropherograms were viewed in Geneious™ version 10.0.9 (Biomatters, Auckland, NZ) and then compared to the published sequence AY492338.1 (Radke et al., 2004) available through GenBank (Benson et al., 2007). For mussel samples, presence of a band of expected size was taken as qualitative evidence of successful amplification of *T. gondii* SporoSAG mRNA, as amplicon products were too weak for successful sequencing.

6.2.8 Statistical analysis

The diagnostic (epidemiological) sensitivity of the nPCR *dhps* assay and true prevalence (as opposed to apparent prevalence given by PCR results) of *T. gondii* were estimated using a Bayesian and Markov Chain Monte Carlo (MCMC) modelling approach (Branscum et al., 2005). Diagnostic specificity was assumed to be 100 % as all positive results were confirmed by direct sequencing. Prior information about the diagnostic sensitivity was modelled using a beta-distribution elicited from a technical expert [L. Howe], assuming a most likely value of 80% and 95% confidence that it is at least 60%. A beta-distribution was also specified for true prevalence informed by overseas prevalence studies, assuming a most likely value of 1.6 % and 95 % confidence that it is less than 50 % (Miller et al., 2008; Esmerini et al., 2010; Putignani et al., 2011; Aksoy et al., 2014; Marangi et al., 2015; Marquis et al., 2015; Shapiro et al., 2015) (Table 17). Analyses were carried out in Betabuster (University of California, Davis, USA) and OpenBUGS version 3.2.3 rev 1012 software to give a posterior median point estimate and 95 % credible interval for both parameters. Elsewhere, 95 % confidence intervals are given using the Wilson method for binomial proportion data. A generalised linear model with a logit link (family = binomial) was used to determine the risk factors for a mussel being *T. gondii* positive. Univariate analyses were performed for source origin (n = 8), year, and season (spring (September, October, November), summer (December, January, February), fall (March, April, May), winter (June, July, August). Interactions were explored with model simplification using X^2 test to compare nested models and odds ratios estimated from the exponent of the coefficients. Low samples sizes and collinear variables caused singularities for models with interaction terms, but the multivariate analyses supported the univariate results. Significance was indicated when $P \leq 0.05$ for all statistical analyses. These analyses were performed in R version 3.4.2 (R Core Team, 2017) using base or plotrix (Lemon, 2006), MASS (Venables & Ripley, 2002) and binom (Dorai-Raj, 2014) packages.

6.3 Results

6.3.1 *Cryptosporidium* spp. and *G. duodenalis* in Commercial Mussels

G. duodenalis DNA was found in 1/90 (1.1 %; 95 % Confidence Interval: 0.06 – 6.9) mussel haemolymph samples, with 99 % nucleotide similarity to *G. duodenalis* Assemblage B at the *gdh* locus (GenBank L40508, Monis et al., 2009). *Cryptosporidium* spp. DNA was not detected in any mussels sampled in the study (Table 15).

6.3.2 Analytical sensitivity and specificity of the nested *dhps* PCR assay for *T. gondii* detection.

Specificity of the primers for detection of *T. gondii* was evaluated by testing DNA of closely related protozoans, *Hammondia hammondi*, *Neospora caninum and Sarcocystis* spp. Gel electrophoresis showed that the nPCR assay specifically amplified the *dhps* gene of *T. gondii* and not the three closely related protozoans. The sensitivity of the assay was determined to be 66.7 % for 50 *T. gondii* oocysts in mussel haemolymph (2/3 PCR replicates) and 100 % for 100 and 1000 oocysts in mussel haemolymph (3/3 PCR replicates).

6.3.3 *T. gondii* in commercial mussels

A total of 13 mussels (12.5 %; 95 % Confidence Interval: 7.09 – 20.8) tested positive for *T. gondii* DNA using the validated nPCR (Table 15). All 13 positive amplicons were confirmed by NCBI BLAST to have 99 – 100 % nucleotide similarity to the *T. gondii dhps* gene (GenBank U81497, Pashley et al., 1997). True prevalence, given by the posterior median point estimate, was calculated to be 16.4 % (95 % Credible Interval: 9.1 – 27.5). Diagnostic sensitivity was calculated to be 77.3 % (95 % Credible Interval: 56.1 – 91.9). A significant difference in *T. gondii* prevalence was observed between mussels sampled in summer, compared with winter, with the odds of detection 15 times higher (95 % Confidence Interval 3 – 113, $P = 0.003$) given the winter baseline (Figure 13). Amplification of SAG1, GRA6, 3'SAG2, and B1

loci was not successful for any of the 13 mussels with confirmed *T. gondii* DNA. Further molecular characterisation using restriction fragment length polymorphism (RFLP) was therefore not possible.

Seven nPCR *dhps* positive mussel samples had sufficient haemolymph remaining for RNA extraction and testing by reverse-transcriptase PCR (RT-PCR). Sporozoite-specific SporoSAG mRNA was detected in four of the seven mussels tested. To ensure that the assay could distinguish between different life-stages of *T. gondii*, the study confirmed that RNA expression of the *T. gondii* SporoSAG gene was limited to sporulated oocysts. Total DNA and RNA extracted from 1000 *T. gondii* tachyzoites and 1000 sporulated oocysts was evaluated. Unsporulated oocysts were not available for this study. An approximately 200 bp fragment was observed in both total DNA samples from the tachyzoites and oocysts. However, an approximately 80 bp band was only observed in the total RNA sample from the 1000 oocysts and not the 1000 tachyzoites. A positive amplicon from total RNA of 1000 oocysts was confirmed by sequencing to have 100 % nucleotide similarity to *T. gondii* sporozoite-specific SAG protein mRNA (GenBank AY492338.1, Radke et al., 2004). The sensitivity of the SporoSAG RT-PCR using total RNA extracted from 1000, 100, 50, 10, 5, 1 sporulated oocysts in PBS, each tested in triplicate, was determined to be 66.7 % for 10 sporulated oocysts (2/3 replicates) and 100 % for 50 sporulated and higher (Appendix 6iii).

Table 15 Apparent prevalence of *T. gondii*, *Cryptosporidium* spp. and *G. duodenalis* DNA in green-lipped mussels (*Perna canaliculus*) collected from eight commercial outlets in New Zealand at seven time points between September 2013 and November 2015. 95 % confidence intervals are given using the Wilson method for binomial proportions. ND, not done

Date Sampled	**No. Positive/No. Tested (%, 95 % Confidence Interval)**		
	Toxoplasma gondii	***Cryptosporidium* spp.**	***Giardia duodenalis***
Sep 2013	4/12 (33.3, 14 - 61)	nd	nd
Mar 2014	0/9 (0.0, 0 - 30)	0/9 (0.0, 0 - 30)	0/9 (0.0, 0 - 30)
Jun 2014	2/11 (18.2, 5 - 48)	0/10 (0.0, 0 - 28)	0/10 (0.0, 0 - 28)
Jul 2014	0/11 (0.0, 0 -26)	0/11 (0.0, 0 - 26)	0/11 (0.0, 0 - 26)
Jan 2015	6/14 (42.9, 21 - 67)	0/14 (0.0, 0 - 22)	0/14 (0.0, 0 - 22)
Jul 2015	0/19 (0.0, 0 - 17)	0/19 (0.0, 0 - 17)	0/19 (0.0, 0 - 17)
Nov 2015	1/28 (3.6, 1 - 18)	0/27 (0.0, 0 - 12)	1/27 (3.7, 1 - 18)
Total	**13/104 (12.5, 8 – 20)**	**0/90 (0.0, 0 - 4)**	**1/90 (1.1, 0 - 6)**

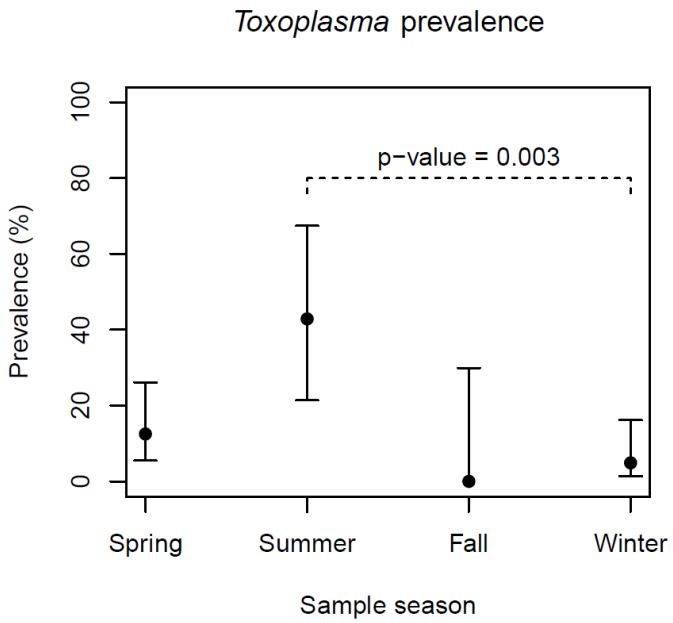
Table 16 Primer sequences, annealing temperatures and expected amplicon size for the four polymerase chain reaction assays used for detection of *Cryptosporidium* spp. DNA, *G. duodenalis* DNA, *T. gondii* DNA and *T. gondii* SporoSAG mRNA in mussel haemolymph

Protozoan	Gene Target	Primer Name	Primer Sequence	Annealing Temp (°C)	Size (bp)	Reference
T. gondii	nPCR *dhps*	FOOD1	GGA ACA TCC GCT GAA GCT CAT GG	57		Aspinall et al., 2002a
		FOOD2	CAG AGA ATC CAG TTG TTT CGA GG			
				57	450	
		FOOD3	CAG TCC AGA CTC GTT CAC CGA TC			
		FOOD4	CCG GAA TAG TGA TAT ACT TGT AG			
	RT-PCR SporoSAG	SporoSAG F	CGG ACA AAT GTG GCG TAC AC	60	71	Travaillé et al., 2016
		SporoSAG R	GTG ATC TTG CGC CGA ACA C			
***Cryptosporidium* spp.**	nPCR GP60	AL3531	ATA GTC TCC GCT GTA TTC	57		Peng et al., 2001, Alves et al., 2003
		AL3535	GGA AGG AAC GAT GTA TCT			
		AL3532	TCC GCT GTA TTC TCA GCC	60	800 - 850	
		AL3534	GCA GAG GAA CCA GCA TC			
G. duodenalis	snPCR *gdh*	GDHeF	TCA ACG TYA AYC GYG GYT TCC GT	56		Read et al., 2004
		GDHiF	CAG TAC AAC TCY GCT CTC GG			
					432	
		GDHiR	GTT RTC CTT GCA CAT CTC C			

Table 17 Prior information about the diagnostic specificity and sensitivity of a nested polymerase chain reaction assay targeting the *dhps* gene for detection of *T. gondii* DNA in mussel haemolymph was modelled using a beta-distribution elicited from a technical expert. A beta-distribution was also specified for true prevalence of *T. gondii* in mussels informed by overseas prevalence studies

Parameter	**Prior estimate**	**5th/95th percentile**
True prevalence	0.016	< 0.5
Sensitivity	0.8	> 0.6
Specificity	1.0	-

Figure 13 Seasonal changes in *Toxoplasma gondii* DNA prevalence in green-lipped mussels collected from commercial sources in New Zealand. Univariate analysis showed that there was a significantly higher prevalence in mussels sampled in the summer months (December, January, February) compared with winter (June, July, August) (P = 0.003). Significance was indicated when P ≤ 0.05. 95% confidence intervals are given using the Wilson method for binomial proportions



6.4 Discussion

This is the first report of zoonotic protozoan parasites, *G. duodenalis* and *T. gondii*, in commercially-sourced green-lipped mussels grown in New Zealand, providing evidence that green-lipped mussels harbour potentially pathogenic protozoan (oo)cysts.

These results suggest that green-lipped mussels may be a good sentinel species for assessing marine contamination with protozoan pathogens in New Zealand. Previous studies have shown that the detection of (oo)cysts in seawater is challenging (Toze et al., 1999; Verant et al., 2014) and an alternative approach is to sample bivalve shellfish as biological sentinels, which has been used to monitor other waterborne pollutants such as heavy metals and biotoxins (Arkush et al., 2003; Lindsay

et al., 2004; Miller et al., 2005b; Shapiro et al., 2015; Kerambrun et al., 2016). Green-lipped mussels can filter up to 9 L of seawater per hour (James et al., 2001) and are able to select food particles on basis of size ranging from 5 – 20 µm (Shumway et al., 1985; Safi & Gibbs, 2003). Thus, (oo)cysts of *G. duodenalis* and *T. gondii*, as well as *Cryptosporidium* spp., are of optimum size for *P. canaliculus*, measuring 10 – 12 µm, 7 – 14 µm, and 4 – 6 µm, respectively (Dubey et al., 1970; Gómez-Couso et al., 2003a; Willis et al., 2013).

Giardia duodenalis DNA was detected in 1 % of mussels tested (1/90, 95 % Confidence Interval: 0 - 6). Molecular characterisation showed that detected DNA belonged to *G. duodenalis* assemblage B, which is known to be pathogenic in humans (Feng & Xiao, 2011). Assemblage B has previously been found in *Ostrea edulis* oysters from Spain (Gómez-Couso et al., 2004) and *Mytilus californianus* mussels from California (Adell et al., 2014). *Giardia* as a genus is made up of six species, but giardiasis in humans and most other mammals is caused by *G. duodenalis,* which is a complex of at least eight genetic assemblages (A – H) (Monis et al., 2003; Feng & Xiao, 2011; Ryan & Cacciò, 2013). So far, only assemblages A and B are known to be pathogenic in humans (Homan et al., 1998; Read et al., 2004) but have been isolated from a variety of other hosts, including livestock, cats, dogs, and wild mammals (Li et al., 2012). In New Zealand assemblage B is the dominant cause of human giardiasis and has been detected in domestic, introduced wild and zoo animals (Garcia-R et al., 2017). Although it is difficult to link protozoal infections to consumption of shellfish, particularly due to long incubation times and lack of symptoms in many people (Robertson, 2007), an outbreak of giardiasis was identified in the United States associated with oyster consumption (Iwamoto et al., 2010) and consumption of recreationally-sourced mussels has been linked to two cases of giardiasis in humans reported in New Zealand between 1997 and August 2004 (Scholes et al., 2009).

The prevalence of *Giardia*-contaminated shellfish reported in this study is relatively low compared with that seen in shellfish in some overseas locations, for example, 10 % of commercial mussels from Italy (*n* = 60, Giangaspero et al., 2014) and 41 % from Spain (*n* = 184, Gómez-Couso et al., 2005) tested positive

for this protozoan. This could be an issue of sample size and sampling frequency but could also be because most studies in other regions test multiple tissues using immunofluorescence antibody assays alone or combined with PCR and may employ immunomagnetic separation to concentrate the parasites in tissue homogenates before testing (Miller et al., 2005; Willis et al., 2013; Adell et al., 2014). It is therefore possible that prevalence was underestimated in this study because only haemolymph was tested and a concentration step was not included.

New Zealand has a higher incidence of human giardiasis than most developed countries, with a notification rate of 32.9 cases per 100,000 population for 2015 (ESR, 2016). The disease is mainly thought to be transmitted from person to person in New Zealand (Snel et al., 2009), although an increasing number of reports show that dairy cattle can act as reservoirs (Hunt et al., 2000, Learmonth et al., 2003; Winkworth et al., 2008; Abeywardena et al., 2012). Cysts are immediately infectious, can rapidly accumulate in the environment, and survive for weeks to months in soil, freshwater and seawater (Brown et al., 1999; Olson et al., 1999; Miller et al., 2005a, b; Robertson, 2007). Thus, the discovery of *Giardia* cysts in freshwater sources in New Zealand (Ionas et al., 1998; Till et al., 2008) and now in green-lipped mussels, suggests that this parasite is being transported in sewage, surface or agricultural runoff to rivers and streams, and eventually to New Zealand coastal waters.

True prevalence of *T. gondii* DNA in mussels tested (*n* = 104) was estimated to be 16.4 %, which was a higher proportion than expected. Mussels were sampled throughout the year over a two-year period. Previous studies that also tested marine shellfish throughout the year have reported a *T. gondii* prevalence of 0.07 % (Miller et al., 2008), 1.4 % (Shapiro et al., 2015), 1.7 % (Marquis et al., 2015) in the USA, 3.3 % (Esmerini et al., 2010) and 8.2 % (Ribeiro et al., 2015) in South America, 3.2 % (Putignani et al., 2011) and 9.4 % in Europe (Aksoy et al., 2014). Seasonal differences have been observed in California, USA, where rainy season sampling appears to be associated with high *T. gondii* prevalence in mussels (Shapiro et al., 2015), even reaching 46. 3 % in a recent study (*n* = 41, Staggs et al., 2015). In locations such as California, USA, most months are dry, and rainfall is needed to flush oocysts into

nearshore waters, resulting in increased levels of marine contamination during the wet season (winter, spring) (Shapiro et al., 2015; VanWormer et al., 2016). Here, in contrast, the odds of detecting *T. gondii* were found to be 15 times higher in mussels sampled in summer, as compared with winter (P = 0.003). On average, New Zealand rainfall is generally higher in winter than in summer (NIWA), so this was an unexpected finding that may be explained by region-specific weather patterns seen in the country (NIWA). It is also possible that higher levels of marine pollution and mussel contamination in summer months were due to drought events followed by intense rainfall. Extended dry periods may lead to greater oocyst accumulation on land that can mobilise into overland runoff with subsequent periods of heavy rain (Shapiro et al., 2012; Lal et al., 2013). Seasonal differences in *T. gondii* prevalence may further be linked to seasonal variations in terrestrial contamination, for example, the timing of the feline breeding season (VanWormer et al., 2013), but this remains to be determined.

Variation in *T. gondii* prevalence between studies may reflect real differences in environmental contamination or seasonal effects. However, it may also be impacted by disparities in test sensitivity, as no standardised protocol for testing shellfish has yet been agreed upon in the literature. Here, a nested PCR targeting the *dhps* gene of *T. gondii* (Aspinall et al., 2002a) was chosen as it has previously been used to investigate *T. gondii* in livestock (Patel et al., 2019) and native wildlife (Roe et al., 2013, 2017; Howe et al., 2014) in New Zealand. Analytical sensitivity of the method for use with mussel haemolymph was established by conducting an oocyst spiking experiment. Only three prior investigations have also used oocyst spiking experiments rather than genomic DNA or DNA from tachyzoites to determine sensitivity (Esmerini et al., 2010; Shapiro et al., 2015; Staggs et al., 2015). Authors report detection limits of 100 oocysts in mussel tissue homogenate (Esmerini et al., 2010), 5 oocysts in mussel haemolymph (Shapiro et al., 2015), and even a single oocyst in mussel haemolymph (Staggs et al., 2015). In comparison, the nPCR *dhps* in this study was able to detect 50 oocysts in haemolymph. Sensitivity differences may be due to a number of factors, particularly PCR target copy number, shellfish species and tissues tested, and nucleic acid extraction methods (Manore et al., 2019). For example, assays targeting the *rep529* gene as used by Staggs et al. (2015) are purportedly the most sensitive (Su et al., 2010) because there are approximately 200 – 300 copies of this marker in the *T.*

gondii genome (Homan et al., 2000, Costa & Bretagne, 2012). It is not yet known whether the *dhps* gene is single- or multi-copy. It is therefore likely that prevalence was underestimated in this study, notably because contaminated shellfish are thought to only contain low oocyst numbers (Esmerini et al., 2010; Shapiro et al., 2015; Staggs et al., 2015).

It is interesting to attempt to estimate true prevalence through use of Bayesian statistics to take into account that true *T. gondii* contamination status of each mussel was not known, and that the PCR assay was not 100 % sensitive (Branscum et al., 2005). Incorporating prior information and observed data to describe the uncertainty surrounding prevalence and sensitivity, the posterior distribution produced an estimate of true prevalence of 16.4 % (95 % Credible Interval: 9.1 – 27.5). Bayesian methods generated an estimate of the mean of the posterior distribution for PCR sensitivity of 77 % (95 % Credible Interval: 56.1 – 91.9). Although the Bayesian true prevalence does not appear to be significantly different from apparent prevalence (12.5 % is found within the credible interval for true prevalence), around a quarter of *T. gondii*-contaminated mussels could yield negative test outcomes using this assay.

The relatively high prevalence of *T. gondii* DNA in green-lipped mussels seen in this investigation may be indicative of a large environmental burden of *T. gondii* oocysts in New Zealand. Cats are the only known source of *T. gondii* oocysts, as the definitive host of the parasite. Sexual replication of *T. gondii* occurs in the cat intestine and during active infection a single cat can shed up to 810 million oocysts into the environment in its faeces, over a period no longer than 3 weeks (Dabritz et al., 2006). Studies in the USA suggest that the environmental load of oocysts depends, in part, on the proportion of owned cats that defecate outdoors (Dabritz & Conrad, 2010). For example, Dabritz & Conrad (2010) predicted that oocyst contamination of soil may be 10 times greater in Central and South America due to 80 % of outdoor cats compared to 8 – 17 % in North America. In New Zealand there is a large owned cat population in excess of 1.1 million (New Zealand Companion Animal Council Inc., 2016) and approximately 61 % of owned cats may be seropositive for *T. gondii* (Chapter 3). It is estimated that at least 90 % of owned cats in the country are allowed outdoors (Farnworth et al., 2010; Hall et al., 2016)

and outdoor cats are likely to have a greater exposure to *T. gondii* through hunting and consumption of birds, rodents and other intermediate hosts, potentially increasing the incidence of cat infections and thus oocyst shedding.

New Zealand also has specific climatic and topographical features which may facilitate oocyst transport from land to sea (VanWormer et al., 2016). Particularly, New Zealand is a long narrow country with a high coastline to land mass ratio. Accordingly, a high proportion of river systems discharge at coastlines rather than at inland water bodies. The climate is generally temperate, with relatively high rainfall (NIWA), thought to drive overland runoff events and transport pathogens to coastal waters (Fayer et al., 2004; Shapiro et al., 2015; VanWormer et al., 2016). Mussels in this study originated from ocean farms so prevalence should reflect marine contamination with *T. gondii*. However, high prevalence could result from cross-transmission of oocysts between mussels in holding tanks, as seen experimentally with *Cryptosporidium* spp. oocysts in shellfish (Gómez-Couso et al., 2003b). The green-lipped mussels used in this study were purchased from local supermarkets where large numbers of mussels are held together. Transmission of contamination may therefore be possible, especially if water is recirculated.

T. gondii infections are regularly reported in terrestrial mammals in New Zealand (e.g. West, 2002; Patel et al., 2017) and have also been reported in marine mammals (Roe et al., 2013, 2017) and birds (Howe et al., 2014). Comparison between genotypes from the terrestrial and marine environments would help clarify routes and mechanisms of land-sea transmission (Fayer et al., 2004; VanWormer et al., 2014). Unfortunately, genotyping was not possible in this study, likely due to low concentrations of parasite DNA in mussel samples and PCR methods lacking the necessary sensitivity to reliably detect low copy numbers of typing loci in shellfish tissues (Esmerini et al., 2010; Shapiro et al., 2015; Staggs et al., 2015).

While the nested *dhps* PCR method used in the current study confirms the presence of *T. gondii* DNA, it does not distinguish sporulated oocysts and other life-stages of *T. gondii*. This is of importance with

respect to disease transmission, since only sporulated *T. gondii* oocysts can be infectious (Dubey et al., 1998). To investigate this, a RT-PCR targeting the SporoSAG gene, which is reported to be sporozoite-specific, was evaluated (Radke et al., 2004; Villegas et al., 2010; Travaillé et al., 2016). Results of this study support findings of prior investigations that conclude the SporoSAG marker is highly expressed in the sporulated oocyst (Radke et al., 2004; Villegas et al., 2010). In the current study the SporoSAG assay was evaluated using RNA extracted from tachyzoites as well as sporulated oocysts, and amplification was only seen using RNA from the oocysts, producing a band of expected size (71 bp), which was confirmed to be SporoSAG mRNA by direct sequencing. A concern with the RT-PCR is whether the assay is amplifying *T. gondii* genomic DNA, which may remain after RNA extraction. The RT-PCR primers were therefore used in a conventional PCR with DNA from both *T. gondii* oocysts and tachyzoites. Amplification was observed but produced a larger band than expected, at 200 bp rather than 71 bp. During assay evaluation with RNA from known quantities of oocysts, however, only bands at ~71 bp were seen, confirming that we were detecting *T. gondii* sporozoites.

The RT-PCR SporoSAG was then successfully used to detect *T. gondii* sporozoite mRNA in four of seven mussels which were positive for *T. gondii* DNA by nPCR *dhps*. As far as the authors are aware, this is the first time this method has been used to confirm the presence of sporulated *T. gondii* oocysts in shellfish. As it is possible to detect SporoSAG mRNA in sporulated but potentially non-viable oocysts (this study; Villegas et al., 2010; Ware et al., 2010; Travaillé et al., 2016), however, we cannot definitively claim that infectious oocysts were found in these mussels. Experimental studies using mouse bioassays have shown that shellfish can ingest and retain viable, infectious oocysts (Arkush et al., 2003; Lindsay et al., 2004). Results of the current investigation suggest that this may also occur in shellfish naturally exposed to *T. gondii* and that the infective stage of the parasite could reach consumers.

In conclusion, green-lipped mussels could serve as bio-indicators of coastal protozoal pollution in New Zealand. Further surveillance using wild mussels should aim to characterise the distribution of these potentially pathogenic organisms in the marine environment and identify associated risk factors. This study appears to be the first to demonstrate that naturally-exposed marine shellfish can harbour

sporulated oocysts of *T. gondii*, providing an additional molecular tool to evaluate health risks for consumers.

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7 Comparison of PCR assays to detect *Toxoplasma gondii* oocysts in green-lipped mussels (*Perna canaliculus*)

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7.1 Introduction

The zoonotic protozoan, *Toxoplasma gondii*, is a ubiquitous terrestrial pathogen that is also recognised as a waterborne parasite (Dubey, 2004; Jones & Dubey, 2010; VanWormer et al., 2014). Human and marine mammal infections with *T. gondii*, thought to be associated with exposure to water or prey contaminated with *T. gondii* oocysts, have been reported globally (e.g. Bowie et al., 1997; De Moura et al., 2006; Dubey et al., 2003; Kreuder et al., 2003; Roe et al., 2013). A growing number of studies have used PCR-based methods to detect *T. gondii* DNA/RNA in naturally-exposed freshwater or marine filter-feeding shellfish (e.g. Esmerini et al., 2010; Shapiro et al., 2015; Staggs et al., 2015; Cong et al., 2017; Ghozzi et al., 2017; Coupe et al., 2018), providing further evidence that this parasite is widespread in aquatic environments.

Toxoplasma gondii is the causative agent of toxoplasmosis, which is one of the most common parasitic infections of humans and other warm-blooded animals, including marine wildlife (Tenter et al., 2000; Batz et al., 2012; Dubey, 2016; Wilking et al., 2016). Although the majority of *T. gondii* infections are thought to be asymptomatic, toxoplasmosis can have severe consequences for infected hosts, and can be fatal (Mead et al., 1999; Tenter et al., 2000; Holland, 2003; Pereira-Chioccola et al., 2009; McLeod et al., 2013). It is thought that *T. gondii* can infect all warm-blooded animals as intermediate hosts, but felids are the only known definitive hosts that can shed oocysts in their faeces (Dubey & Frenkel, 1972). Once in the environment, *T. gondii* oocysts sporulate to become infectious, within one to five days of excretion depending upon aeration and temperature (Dubey et al., 1998). Sporulated oocysts are extremely hardy, able to survive in soil, freshwater, and saltwater for over a year (Frenkel et al., 1975; Dubey, 1998; Lindsay et al., 2003; Lindsay & Dubey, 2009), and can pollute freshwater and marine environments, surviving transport to the coast in land-sea runoff (Miller et al., 2002; Conrad et al., 2005; VanWormer et al., 2014). In New Zealand, recent work has shown that *T. gondii* is present in coastal ecosystems (Chapter 6; Coupe et al., 2018), and toxoplasmosis has been identified as a cause of mortality for some marine mammal species in the country (Roe et al., 2013, 2017).

Despite the risks that coastal contamination with *T. gondii* oocysts may pose for human and marine wildlife health, at present there are no commercially-available methods to concentrate *T. gondii* oocysts directly from seawater, and molecular confirmation of *T. gondii* in environmental seawater samples has not yet been successful (Jones & Dubey, 2010; Shapiro et al., 2010, 2015; Verant et al., 2014). However, filter-feeding shellfish, such as mussels, oysters, and clams, may serve as biosentinels by which to monitor the extent of *T. gondii* pollution in marine ecosystems (Palos Ladeiro et al., 2014; Shapiro et al., 2015; Staggs et al., 2015; Coupe et al., 2018). Studies have shown that shellfish can filter and accumulate sporulated oocysts of *T. gondii* (Arkush et al., 2003; Lindsay et al., 2004; Coupe et al., 2018), and a variety of PCR-based molecular methods have confirmed the presence of *T. gondii* nucleic acids in several naturally-exposed shellfish species (Miller et al., 2008; Esmerini et al., 2010; Putignani et al., 2011; Aksoy et al., 2014; Shapiro et al., 2015; Staggs et al., 2015; Cong et al., 2017; Coupe et al., 2018). Yet, there is no standardised PCR method available for detecting *T. gondii* in shellfish (Hohweyer, 2013; Shapiro et al., 2015), and there are only a few studies that compare sensitivity and specificity of these assays for this purpose (Arkush et al., 2003; Putignani et al., 2011; Shapiro et al., 2015; Staggs et al., 2015).

Thus, the primary aim of this study was to evaluate the analytical sensitivity and specificity of four commonly used molecular assays for detection of *T. gondii* tachyzoites and oocysts. Two nPCR assays targeting either the *dhps* (Pashley et al., 1997) or B1 gene (Burg et al., 1989), and two qPCR assays targeting either the B1 gene or a 529 bp repetitive element (Homan et al., 2000) were selected. Additionally, these assays were validated for *T. gondii* detection in green-lipped mussel haemolymph using oocyst spiking experiments to assess their usefulness for *T. gondii* surveillance in coastal ecosystems.

7.2 Methods

7.2.1 *Toxoplasma gondii* tachyzoites and oocysts

Live, attenuated, tachyzoites of Type I S48 strain were obtained from the commercial Toxovax® vaccine (Schering-Plough Animal Health, Wellington, New Zealand) (Hartley & Bridge, 1975). *Toxoplasma gondii* oocysts (Type II M4 strain, originally obtained from Lee Innes of the Moredun Research Institute, Edinburgh, Scotland) were provided by Heather Fritz, Jeroen Saeij, and David Arranz Solis (University of California, Davis). Oocysts were not purified but had been heat inactivated by immersion in an 80 °C dry bath for 20 min.

7.2.2 *Toxoplasma gondii* stock preparation

The concentration of stock solutions of tachyzoites and oocysts was determined using a haemocytometer chamber and light or epifluorescence microscopy, respectively. Suspensions of 1000, 100, 10, 1 tachyzoites and 1000, 100, 50, 10, 5, 1 sporulated oocysts were then prepared by serial dilution with sterile phosphate-buffered saline (PBS) (1X, pH 7.4) and used as PCR controls and to evaluate the analytical sensitivity of the PCR assays. Samples were centrifuged at 20,000 ×*g* for 10 min, the supernatant removed, and the cell pellet suspended in 100 µl PBS (1×, pH 7.4).

7.2.3 Haemolymph spiking with *T. gondii* oocysts

Haemolymph was obtained from commercial green-lipped mussels previously determined to be negative for *T. gondii* DNA in duplicate PCR tests. Hemolymph from several mussels was pooled and divided into 100 µl aliquots. Each 100 µl aliquot was spiked with serial dilutions of *T. gondii* oocysts before the haemolymph was pelleted, supernatant aspirated, and cell pellet suspended, as above.

7.2.4 DNA extraction

DNA was extracted from serially diluted tachyzoites, oocysts, and oocyst-spiked haemolymph, using Qiagen DNeasy Blood and Tissue kits® (Qiagen, Hilden, Germany). For tachyzoites, DNA was extracted according to the manufacturer's instructions for non-nucleated blood, including an overnight digest at 56 °C with proteinase K. For oocysts and oocyst-spiked haemolymph, DNA was extracted following procedure detailed in Shapiro et al. (2015). Briefly, ATL buffer (180 µl) was added to each sample before one freeze-thaw cycle of immersion in liquid nitrogen (4 min)/boiling water (4 min). Proteinase K (40 µl) was then added before incubation overnight at 56 °C. After addition of Buffer AL (200 µl), samples were incubated at 70 °C for 10 min. DNA was eluted in 10 % AE solution (50 µl), which was first heated to 95 °C. After application of elution buffer, spin columns were incubated at room temperature for 5 min before being centrifuged (8,000 x*g*, 2 min). A non-spiked haemolymph and PBS only samples were included to serve as additional negative controls in the DNA extractions and subsequent PCR reactions. DNA was extracted for each parasite concentration and stored at -80 °C until molecular analysis.

7.2.5 PCR protocols

7.2.5.1 nPCR protocols

Two nPCR assays were evaluated, one using primers targeting the dihydropteroate synthase (*dhps*) gene (Pashley et al., 1997), and the other targeting the B1 gene (Burg et al., 1989). The nPCR *dhps* assay targeted a 450 bp fragment of the *T. gondii dhps* gene using FOOD1/2 and FOOD3/4 primers (Aspinall et al., 2002), as described by Roe et al. (2013) (Table 18). For detection of *T. gondii* oocyst DNA, bovine serum albumin (BSA, 10 mg/ml) was added to each PCR reaction mix to minimise effects of PCR inhibitors. The nPCR B1 targeted a 530 bp fragment of the *T. gondii* B1 gene using Pml/S1, Pml/AS1, Pml/S2, and Pml/AS2 primers, as described by Grigg and Boothroyd (2001), with the addition of BSA (1 mg/ml) to each PCR reaction mix (Shapiro et al., 2015) (Table 18). All PCR reactions were performed using a conventional PCR thermal cycler (Veriti 96 Well Thermal Cycler, Applied Biosystems Inc, CA, USA) and each PCR assay was run in triplicate per parasite concentration. To confirm successful amplification, 10 µl of the final PCR product was run on a 1.5 % agarose gel stained with SYBR Safe

(Thermo Fisher Scientific), before visualisation by UV light using an E-Gel Imager (Life Technologies, Carlsbad, CA, USA). Presence of a band of expected size, consistent with that of the positive control, was taken as qualitative evidence of successful amplification of *T. gondii* DNA. The size of the PCR amplicon was estimated by comparison with a 100 bp DNA ladder (Promega, Madison WI, USA). Controls included DNA extracted from a known *T. gondii* isolate (incomplete strain S48, Toxovax®) and ultrapure water as a negative control. Assay specificity was verified using DNA extracted from closely related protozoans, *Hammondia hammondi*, *Neospora caninum* and *Sarcocystis* spp., whose identities had been previously confirmed by sequencing. *Hammondia hammondi* DNA was available from a feline faecal float with microscopically-observed *Hammondia*-like oocysts. *Sarcocystis* spp. DNA was available from alpaca skeletal tissue with observable *Sarcocystis* spp. bradyzoite stages. *Neospora caninum* DNA was available from cell cultured *N. caninum* tachyzoites, originally sourced from a calf brain (Okeoma et al., 2004).

7.2.5.2 qPCR protocols

Two genomic targets were compared using qPCR, the B1 gene and the 529 bp repeat element (rep529) (Homan et al., 2000). Primers used for the qPCR B1 were oligo1 and oligo4, as described by Burg et al. (1989), producing a target amplicon of 193 bp (Table 18) (Appendix 7ii). Primers used for the rep529 qPCR were ToxoRE_f and ToxoRE_r, as described by Kasper et al. (2009), with slight modifications, producing a target amplicon of 81 bp (Table 18). Probes were not included in either qPCR assay. Targets were amplified using FastStart Universal SYBR Green Master (ROX) (Roche, Manheim, Germany) two-step method. Final reaction mixtures for tachyzoite experiments (20 µl total) included 10 µl 2X FastStart Universal SYBR Green Master (ROX), 0.25 µM forward primer, 0.25 µM reverse primer, and 2 µl of template DNA. Final reaction mixtures for oocyst experiments (10 µl total) included 5 µl 2X FastStart Universal SYBR Green Master (ROX), 0.25 µM forward primer, 0.25 µM reverse primer, 0.5 µl BSA (10 mg/mL), and 2 µl of template DNA. A Mic qPCR cycler (Bio Molecular Systems, Queensland, Australia) was used for all qPCR analyses. Amplification conditions were 95 °C 15 min followed by 40 cycles at 95 °C for 15 sec, 53 °C for 30 sec (B1) or 54 °C for 30 sec (rep529), and 72 °C for 30 sec. Each reaction was followed by a melting curve 70 to 85 °C, 0.1 °C per sec. Standard curves and reaction efficiencies were

calculated for each run using micPCR version 2.6.4 (Bio Molecular Systems). A run was considered valid if the qPCR efficiency was > 90 %. Correlation between parasite concentration and Cq values was considered to be 'poor' if the R2 value < 0.90, 'fairly good' if 0.90 ≤ R2 < 0.95, 'good' if 0.95 ≤ R2 < 0.98, and 'excellent' if R2 ≥ 0.98. Assay specificity was further verified using DNA extracted from closely related protozoans, as described above. Positive and negative controls as described above were also included for all qPCR runs.

7.3 Results

7.3.1 Comparison of PCR performance using DNA from *T. gondii* tachyzoites

Sensitivities of the four assays were initially assessed using DNA extracted from known quantities of tachyzoites. Tachyzoites were used for initial assay optimisation as they were readily available, whereas oocysts were not immediately available due to the need for animal infection experiments for their production. All four PCR assays were able to detect DNA from 1000 to 10 tachyzoites, but the nPCR B1 was most sensitive, able to consistently detect DNA from a single tachyzoite (Table 19). The limit of detection of the nPCR *dhps* assay was found to be 10 tachyzoites. Sensitivity was determined to be 66.7 % for 10 *T. gondii* tachyzoites (2/3 PCR replicates) and 100 % for 100 and 1000 tachyzoites (3/3 PCR replicates), whereas the limit of detection of the nPCR B1 was 1 tachyzoite (3/3 replicates). For both qPCR assays, there was good correlation (R^2 = 0.97) between Cq values and tachyzoite concentration, although the limit of detection for both assays was higher than the B1 nPCR, at 10 tachyzoites (3/3 replicates) (Table 19). Melt curve analysis revealed that PCR products from the qPCR B1 had melting temperatures of 79.7 ± 0.2 °C (Fig. 14A). The qPCR B1 did amplify DNA from a single tachyzoite (1/3 replicates) but the melting temperature of this product was outside of the predicted temperature range, at 78.3 °C, and therefore was considered non-specific. Melt curve analysis of the rep529 qPCR assay showed a single peak between 80.0 ± 0.1 °C for all amplicons from tachyzoites (Fig. 14B).

7.3.2 Comparison of PCR performance using DNA from *T. gondii* oocysts

Sensitivities of the four assays were further assessed using DNA extracted from known quantities of *T. gondii* oocysts (Table 19). Although sensitivities varied between the assays, all could consistently detect DNA extracted from 50 – 1000 oocysts. The rep529 qPCR was most sensitive, able to consistently detect DNA from as few as 5 oocysts. The nPCR *dhps* could only reliably detect 50 oocysts or higher (3/3 replicates). However, a lower limit of detection was achieved with the nPCR B1, which had a sensitivity of 66.7 % for 10 oocysts (2/3 replicates) and 5 oocysts (2/3 replicates). The qPCR B1 could also only reliably detect 50 oocysts (3/3 replicates), with a sensitivity of 33.3 % for 10 and 5 oocysts (1/3 replicates) (Table 19). Although there was good agreement (R^2 = 0.97) between Cq values and oocyst dilutions for the qPCR B1, PCR efficiency was poor, only reaching 70 %. Most qPCR B1 amplicons had consistent melting temperatures of 80.1 ± 0.2 °C which was a slight shift to the right from the melting temperatures observed with the tachyzoite amplicons (Fig. 14A). The rep529 qPCR assay was most sensitive, amplifying all three replicates of 5 oocysts and above (Table 19). There was good agreement (R^2 = 0.97) between Cq values and oocyst dilutions for this assay, with a single melt curve peak between 80.0 ± 0.1 °C (Fig. 14B).

7.3.3 Comparison of PCR performance using DNA from haemolymph spiked with *T. gondii* oocysts

Finally, the four PCR assays were evaluated for the detection of DNA from *T. gondii* oocysts spiked into green-lipped mussel haemolymph (Table 20). All four assays consistently detected 50 oocysts spiked into mussel haemolymph (3/3 replicates). The qPCR assays were most sensitive, with a limit of detection of 5 oocysts, whereas both nPCR assays failed to amplify DNA at lower oocyst concentrations. Although the qPCR B1 was relatively sensitive, agreement between Cq values and number of spiked oocysts was poor (R^2 = 0.73), and efficiency was excessively high (147 %) when detecting *T. gondii* DNA from 10 and 5 spiked oocysts for a single replicate. Melt curves for qPCR B1 amplicons from 50 spiked oocysts or higher produced peaks between 80.0 ± 0.1 °C (Fig. 14A). For amplicons from less than 10 spiked oocysts, only the non-specific melt curve peak was observed. The rep529 qPCR assay was able to

detect DNA from 10 and 5 spiked oocysts for a single replicate with good agreement (R^2 = 0.97) and consistent melt curve peaks between 79.9 ± 0.1 °C (Fig. 14B), although PCR efficiency was reduced to 82 %.

7.3.4 Evaluation of primer specificity

When specificity of the protocols was tested against other apicomplexan parasites, the nPCR assays were the most specific, detecting only *T. gondii* DNA, while both qPCR assays cross-reacted with *Sarcocystis* spp. The rep529 qPCR also cross-reacted with *Neospora caninum* (Fig. 14D). The qPCR B1 amplified *Hammondia hammondi* and *Neospora caninum* DNA but the PCR products had melting temperature peaks at 81.8 °C and 78.1 °C, respectively (Fig. 14C), and so could be distinguished from peaks generated from *T. gondii* target DNA.

7.4 Discussion

Due to a lack of efficient and standardised methods for direct detection of *T. gondii* oocysts in seawater, testing of filter-feeding shellfish as biosentinels of aquatic ecosystem pollution has been advocated as an alternative surveillance strategy (Palos Ladeiro et al., 2014; Shapiro et al., 2015; Staggs et al., 2015; Kerambrun et al., 2016; Coupe et al., 2018). Many molecular assays have been developed for the specific detection of *T. gondii* in biological samples (Su et al., 2010; Bahia-Oliviera et al., 2017) and several have now been adapted for use in shellfish, however method standardisation is scarce (Shapiro et al., 2015; Bahia-Oliviera et al., 2017). The B1 gene is the most widely used PCR target in shellfish studies (Arkush et al., 2003; Esmerini et al., 2010; Putignani et al., 2011; Aksoy et al., 2014; Marangi et al., 2015; Marquis et al., 2015; Shapiro et al., 2015; Cong et al., 2017; Ghozzi et al., 2017), followed by the rep529 marker (Palos Ladeiro et al., 2014; Ribeiro et al., 2015; Staggs et al., 2015; Kerambrun et al., 2016), in both conventional and qPCR assays. Other PCR targets include the 18S rRNA (Arkush et al., 2003; Miller et al., 2008), ITS-1 (Zhang et al., 2014; Shapiro et al., 2015), SAG1 (Ribeiro et al., 2015; Shapiro et al., 2015), and *dhps* (Coupe et al., 2018; this study) genes. Despite the variety, there appears to be a consensus in the *T. gondii* literature that PCR assays based upon the rep529

marker generally perform best in terms of sensitivity, independently of the DNA primers and PCR technology used, and also sample type (Edvinsson et al., 2006; Kasper et al., 2009; Yang et al., 2009; Sterkers et al., 2010; Su et al., 2010; Staggs et al., 2015; Wells et al., 2015). Indeed, the rep529 qPCR assay described by Kasper et al. (2009) yielded a sensitivity of 1/30 to 1/50 of a single parasite genome (assuming there are 200 – 300 copies of the rep529 marker in the *T. gondii* genome) per PCR reaction, determined using a plasmid standard dilution series. With respect to shellfish, few prior studies have used oocyst spiking experiments to assess PCR performance (Esmerini et al., 2010; Shapiro et al., 2015; Staggs et al., 2015), and authors report varied sensitivities. Using a nPCR assay targeting the B1 gene, Esmerini et al. (2010) reported detection limits of 1000 and 100 oocysts in tissue homogenates from mussels and oysters, respectively. A detection limit of 5 oocysts in mussel haemolymph was achieved by Shapiro et al. (2015), also using a nPCR assay targeting the B1 gene, as well with nPCR assays targeting the ITS-1 gene, and the rep529 marker. Both the rep529 conventional and qPCR assays reported by Staggs et al. (2015) could consistently detect a single oocyst spiked into mussel haemolymph, currently representing the most sensitive molecular assays described in shellfish.

In the present study, the performance of four previously published PCR assays was compared using *T. gondii* tachyzoites, oocysts, and oocyst-mussel spiking experiments. The primary aim of the study was to determine the best-performing assay for detection of *T. gondii* oocyst DNA in the green-lipped mussel, as a potential bioindicator of *T. gondii* pollution in marine ecosystems in New Zealand. Spiking experiments showed that the rep529 qPCR provided the best sensitivity for detection of *T. gondii* oocyst DNA in the green-lipped mussel, with good correlation between oocyst concentrations and Cq values and a detection limit of 5 spiked oocysts, comparable to previous spiking studies (Shapiro et al., 2015). Although the qPCR B1 provided a detection limit of 5 spiked oocysts, this assay performed poorly in terms of efficiency, and the correlation between oocysts numbers and Cq values was low. Non-specific amplification was also observed, even after assay optimisation, so its use for detection of *T. gondii* oocysts in shellfish is probably limited. Both nPCR assays consistently detected 50 spiked oocysts, but not fewer. Spiking experiment results, therefore, appear to support previous findings that the

rep529 target offers a sensitive tool for detection of *T. gondii* oocyst DNA in shellfish (Staggs et al., 2015).

Interestingly, results demonstrated that the nPCR B1 proved to be most sensitive when the assay was evaluated using tachyzoites, with amplification of DNA from a single parasite, compared with the rep529 qPCR (and other assays) which amplified DNA from 10 tachyzoites. While oocysts are the hardy life-stage of *T. gondii* that can survive in the environment and accumulate in shellfish, it is the tachyzoite and bradyzoite (in tissue cysts) life stages that are found in tissues of infected warm-blooded hosts. At present, the nPCR *dhps* evaluated in this study is favoured for molecular confirmation of toxoplasmosis in infected warm-blooded hosts in New Zealand (Roe et al., 2013, 2017; Howe et al., 2014; Patel, 2016). Results suggest that, although the rep529 qPCR performed best to detect *T. gondii* oocysts in green-lipped mussels, the nPCR B1 may be preferable for testing infected intermediate hosts due to its superior sensitivity using tachyzoite DNA. This assay is also dual-purpose, as the B1 locus can be used for genotyping (Grigg & Boothroyd, 2001; Shapiro et al., 2015).

The rep529 qPCR was considered to be the most sensitive assay for testing green-lipped mussels in this study. The increased sensitivity of rep529-based PCR assays could be due to differences in PCR target copy numbers. Both the rep529 and the B1 gene are multi-copy, but there are up to 20 times as many copies of the rep529 marker in the *T. gondii* genome, compared to the B1 (Burg et al., 1989; Homan et al., 2000; Reischl et al., 2003; Costa & Bretagne, 2012), while the copy number of the *dhps* gene remains to be determined. The sensitivity of the rep529 qPCR, however, cannot fully be explained by differences in PCR target copy number, as the nPCR B1 provided the best sensitivity for detection of tachyzoite DNA, and was also comparable to the rep529 qPCR for detection of DNA from free oocysts. Results of the present study highlight the importance of using oocyst spiking experiments in determining assay sensitivity, particularly because of the presence of PCR inhibitors (Staggs et al., 2015; Kerambrun et al., 2016), which in shellfish tissues can include glycogen and acidic polysaccharides (Schwab et al., 1998). Haemolymph seems to be the least inhibitory tissue of shellfish for molecular

testing, as it is a less dense and complex matrix than gill or digestive gland (Esmerini et al., 2010; Palos Ladeiro et al., 2015; Shapiro et al., 2015; Staggs et al., 2015). Nevertheless, all four assays evaluated in this study performed better when applied on DNA from tachyzoites and free oocysts in PBS as compared with oocysts spiked into mussel haemolymph, despite the addition of BSA to PCR reactions (Jiang et al., 2005). Results showed that when applied on oocysts-spiked in haemolymph, the assays suffered reduced sensitivity, or reduced qPCR assay efficiency/R^2 values, or both. Particularly, the sensitivity of the nPCR B1 was reduced 10-fold from 5 to 50 oocysts when oocysts were spiked into haemolymph. The traditional approach for determining assay sensitivity has been to use plasmids or dilution series of genomic DNA from tachyzoites (Staggs et al., 2015). Yet, results indicate that if we had taken this approach, we would have concluded that the nPCR B1 performed better than the rep529 qPCR, whereas the nPCR B1 seemed to be particularly affected by PCR inhibitors specific to green-lipped mussel haemolymph in this study. Use of the nPCR B1 assay in shellfish surveillance studies in New Zealand may therefore underestimate *T. gondii* prevalence, although further research is needed.

The use of oocysts to validate methods for *T. gondii* detection in environmental samples is also important because a single sporulated oocyst contains eight individual parasites (sporozoites), i.e. eight *T. gondii* genomes, whereas one tachyzoite contains a single genome (Dubey et al., 1998), so comparison of PCR assay sensitivities between studies using plasmids or different life-stages of *T. gondii* may be misleading. Furthermore, the oocyst life-stage is particularly resistant to environmental stressors, requiring freeze-thaw cycles or bead-beating to fracture the tough oocyst wall (Hohweyer, 2013), which may impact DNA extraction, and subsequent molecular analyses.

While the rep529bp assay appeared to perform well compared with other assays previously evaluated in shellfish, it is difficult to compare assay performance across studies. This is not only because authors use different PCR targets, primers, and amplification conditions, but also because of protocol variations that may influence detection limits, such as the type of standard samples used and shellfish tissue tested. The application of different DNA/RNA extraction methods will further influence PCR detection

limits (Bastien et al., 2008), as well as differences in inhibitor removal abilities between preparations (Staggs et al., 2015). For example, the rep529-based PCR assay described by Staggs et al. (2015) are reportedly the most sensitive assays overall for testing shellfish for *T. gondii*, as they were able to consistently detect a single spiked oocyst. The increased sensitivity achieved by Staggs et al., may be due to the fact that oocysts used here were not purified, to better mimic natural conditions, and because the detection limit was based on the serial dilution of a haemocytometer-counted suspension, which may be less accurate than fluorescence activated cell sorting. However, it may also be due to differences in DNA extraction methods between studies. Notably, the Staggs protocol spiked oocysts into haemolymph samples after the haemolymph had been pelleted, whereas in this study (following Shapiro et al., 2015), oocysts were spiked into haemolymph samples before they were pelleted, again to better mimic natural conditions. Sensitivity was reduced to 10 oocysts for Staggs et al. when results were re-evaluated using an alternative DNA extraction protocol. In summary, there may be tissue- and possibly species-specific PCR inhibitors present in shellfish which could affect PCR performance. Therefore, spiking experiments are required to evaluate matrix-specific inhibition, as well as protocols for preparation of nucleic acids prior to PCR, taking into account that there may be a trade-off between sensitivity and removal of PCR inhibitors (Schrader et al., 2012; Staggs et al., 2015).

Maximum sensitivity is desired for *T. gondii* detection in shellfish because there are likely to be low oocyst numbers in shellfish tissues (< 100 parasites per mussel) (Hohweyer, 2013; Aksoy et al., 2014; Marangi et al., 2015). However, it is also important that PCR assays are specific, to minimise false positive results. Previous work claims that the highly sensitive rep529 marker is also highly specific to *T. gondii* (Homan et al., 2000; Kasper et al., 2009), but results of the present study indicate that this is not always the case. Particularly, we found that the rep529 primers (Kasper et al., 2009) evaluated in this study cross-reacted with *Sarcocystis* spp. and *N. caninum* DNA, both protozoans that are closely related to *T. gondii*, which may also contaminate marine ecosystems (Dubey et al., 2003; Miller et al., 2010; Michaels et al., 2016). Results suggest that it may be necessary to incorporate the associated probe of Kasper et al. (2009) (ToxoRE_p (FAM-5'-CTA CAG ACG CGA TGC C-3'-NFQ-MGB; FAM, 6-carboxy-fluorescin; NFQ-MGB, nonfluorescent quencher plus attached MGB), although specificity of the primer

and probe set was not assessed by the authors using DNA from related protozoans. Kasper et al. (2009) also reported using a higher annealing temperature (62 °C) to ensure 100 % specificity, determined using the primer pair in a SYBR Green qPCR assay. However, in the present study an annealing temperature of 62 °C gave rise to dual bands on conventional temperature gradient PCR, and dual melt curve peaks on qPCR, with a weaker band or smaller peak at the expected amplicon size or melt temperature, respectively, a phenomenon also observed by Yang et al. (2009) (using a different set of rep529 primers, from Reischl et al. (2003)). An annealing temperature of 54 °C was optimal for the rep529 primers used in this study. Results also confirm that false positive amplification can occur with other common PCR targets, as the qPCR B1 cross-reacted with *Sarcocystis* spp. DNA. A recent study by Shapiro et al. (2015) found that ITS-1 and B1 primer sets generated a high proportion of false positives, due to the presence of DNA from mussels, amoeba, algae, phytoplankton, as well as other related protozoans. Therefore, the rep529 qPCR in this study may serve as a sensitive screening assay for use with green-lipped mussels, but sequence analysis will be required for definitive confirmation of *T. gondii* DNA (Shapiro et al., 2015; Bahia-Oliviera et al., 2017).

In conclusion, the rep529 qPCR used in this study was found to be a suitably sensitive assay to detect the low numbers of oocysts expected in naturally-exposed shellfish. However, an important finding drawn from the study is that primers/PCR targets thought to be highly specific to *T. gondii*, including the rep529 marker, may in fact cross amplify non-target organisms, confirming the importance of direct sequencing of PCR products as a confirmatory test. Particularly, this study confirmed that oocyst spiking experiments are a vital component of PCR validation and assay comparison, as assays may be impacted to varying degrees by inhibitors present in shellfish tissues, which will affect prevalence estimates in surveillance studies. In summary, assays for detection of *T. gondii* in environmental matrices should be carefully selected based on study aims, targeted parasite life-stage, and sample type to be tested, and guided by assay validation procedures for specific experimental conditions and matrix types.

Table 18 Primer sequences, annealing temperatures, and expected amplicon size for the four polymerase chain reaction assays used for detection of *Toxoplasma gondii* DNA

PCR Type	Gene Target	Primer Name	Primer Sequence 5' – 3'	Annealing Temp (°C)	Size (bp)	Reference
nPCR	*dhps*	FOOD1	GGA ACA TCC GCT GAA GCT CAT GG	57	494	Aspinall et al. (2002)
		FOOD2	CAG AGA ATC CAG TTG TTT CGA GG	57	450	
		FOOD3	CAG TCC AGA CTC GTT CAC CGA TC			
		FOOD4	CCG GAA TAG TGA TAT ACT TGT AG			
	B1	Pml/S1	TGT TCT GTC CTA TCG CAA CG	60	579	Grigg and Boothroyd (2001)
		Pml/AS1	TCT TCC CAG ACG TGG ATT TC			
		Pml/S2	ACG GAT GCA GTT CCT TTC TG	60	530	
		Pml/AS2	CTC GAC AAT ACG CTG CTT GA			
qPCR	B1	oligo1	GGA ACT GCA TCC GTT CAT GAG	53	193	Burg et al. (1989)
		oligo4	TCT TTA AAG CGT TCG TGG TC			
	529 bp Repeat Element (rep529)	ToxoRE_f	CAC AGA AGG GAC AGA AGT CG	54	81	Kasper et al. (2009)
		ToxoRE_r	CAG TCC TGA TAT CTC TCC TCC AAG			

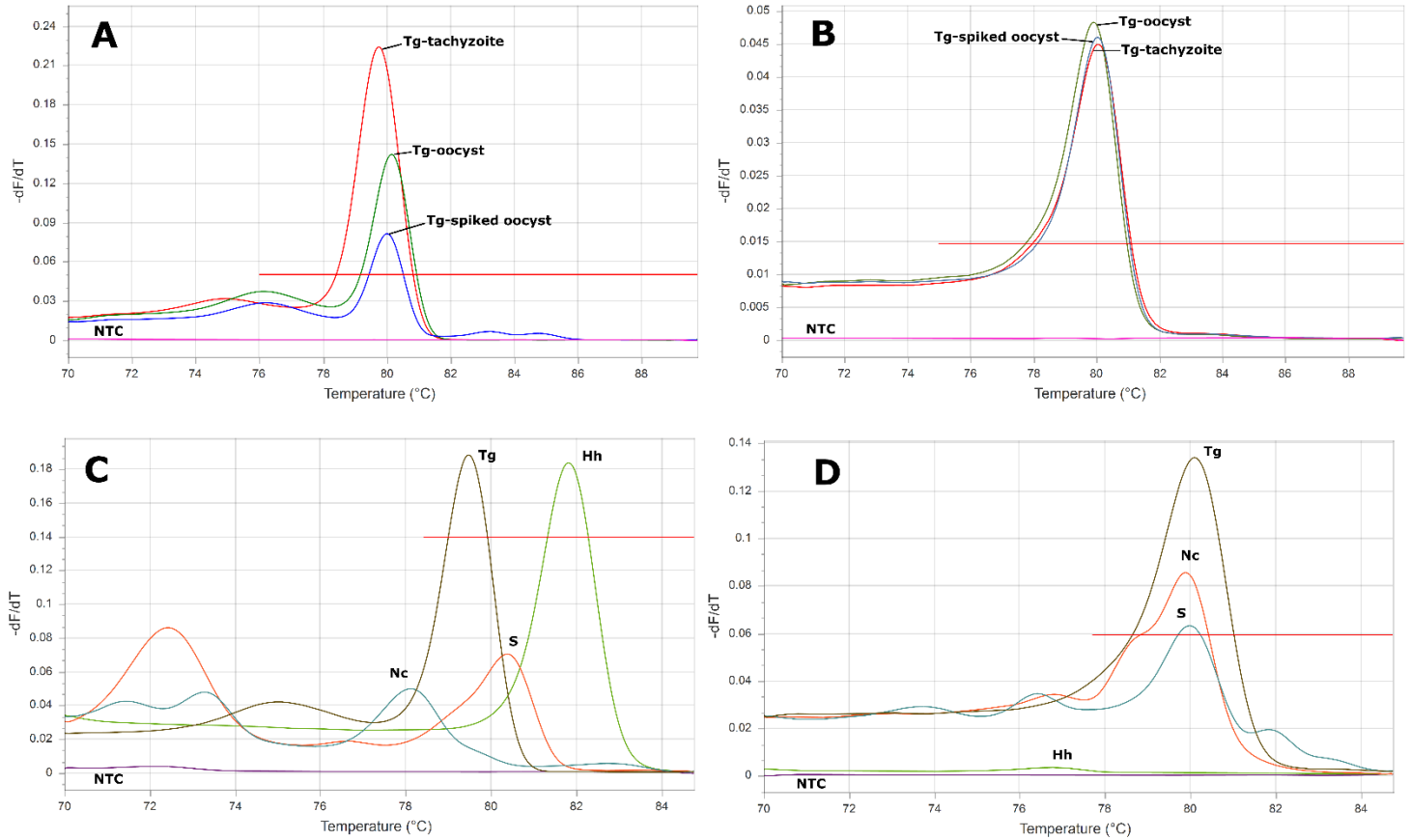
Table 19 Sensitivity of four PCR methods for detection of *Toxoplasma gondii* DNA from known quantities of *T. gondii* tachyzoites and oocysts

	nPCR		qPCR	
No. tachyzoites	***dhps***	**B1**	**B1**	**RE**
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
10	2/3	3/3	3/3	3/3
1	0/3	3/3	0/3	0/3
0	0/3	0/3	0/3	0/3
No. oocysts	***dhps***	**B1**	**B1**	**RE**
1000	3/3	3/3	3/3	3/3
100	2/3	3/3	3/3	3/3
50	3/3	3/3	3/3	3/3
10	0/3	2/3	1/3	3/3
5	0/3	2/3	1/3	3/3
1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

Table 20 Sensitivity of four PCR methods for detection of *Toxoplasma gondii* DNA from known quantities of *T. gondii* oocysts spiked into mussel haemolymph

	nPCR		qPCR	
No. oocysts	***dhps***	**B1**	**B1**	**RE**
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
50	2/3	3/3	3/3	3/3
10	0/3	0/3	1/3	1/3
5	0/3	0/3	1/3	1/3
1	0/3	0/3	0/3	0/3
0	-	-	-	-

Figure 14 Melt curves from qPCR amplification of the B1 gene (**A**, **C**) and rep529 marker (**B**, **D**) of *Toxoplasma gondii*. **A**, **B** using DNA extracted from known quantities of tachyzoites (Tg-tachyzoite), oocysts (Tg-oocyst), and oocyst-spiked green-lipped mussel (*Perna canaliculus*) haemolymph (Tg-spiked oocyst). **C**, **D** using DNA extracted from related protozoa, previously confirmed by sequencing. Tg, *Toxoplasma gondii* positive control; NTC, no template control (ultrapure water); Hh, *Hammondia hammondi*; Nc, *Neospora caninum*; S, *Sarcocystis* spp.



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8 Surveillance for *Toxoplasma gondii* in wild New Zealand shellfish: a preliminary field study in the Waikato Region

8.1 Introduction

In New Zealand, toxoplasmosis has been identified as an important cause of mortality for the endangered Hector's dolphin (*Cephalorhynchus hectori*), including the critically endangered Maui dolphin (*Cephalorhynchus hectori mauii)* subspecies (Roe et al., 2013). The exact mode of transmission is not known, but infections in marine mammals like dolphins are thought to occur through ingestion of *Toxoplasma gondii* oocysts, that enter the marine environment in freshwater runoff contaminated by cat faeces (Cole et al. 2000; Johnson et al. 2009; Miller et al. 2002; Shapiro et al. 2015). To better understand *T. gondii* transmission in the marine environment, it is necessary to investigate the extent of coastal contamination with the parasite and to compare molecular genotypes of *T. gondii* on land and in coastal waters (Miller et al. 2008; Shapiro et al. 2015; VanWormer et al. 2014). It is also important to investigate risk factors for marine contamination with *T. gondii* oocysts as variables associated with an increased risk of toxoplasmosis in Hector's dolphins have not yet been fully clarified (Roe et al. 2013; Chapter 2).

Direct testing for *T. gondii* in marine ecosystems is technically difficult (Shapiro et al., 2019, 2010; Verant et al., 2014) and although *T. gondii*-like oocysts have been observed in environmental samples of seawater (Verant et al. 2014), molecular confirmation has not yet been successful. It is also challenging to obtain sufficient data from coastal marine mammals that can serve as sentinel species of nearshore pathogen contamination, as long-term live or post-mortem studies of these species are often scarce and can face diagnostic difficulties (van de Velde et al. 2016; Chapter 2). An alternative strategy is to target filter-feeding bivalves (Shapiro et al., 2015; Staggs et al., 2015). Shellfish and other filter-feeding invertebrates can accumulate *T. gondii* oocysts from seawater (Lindsay et al., 2004) and testing has found marine ecosystem

contamination with the parasite in various countries, including the USA (Miller et al. 2008; Shapiro et al. 2015; Staggs et al. 2015), Turkey (Aksoy et al., 2014), Brazil (Esmerini et al. 2010; Ribeiro et al. 2015), China (Zhang et al., 2014), and New Zealand (Coupe et al. 2018, Chapter 6). As molecular methods are used to test shellfish tissues for the presence of *T. gondii* nucleic acids, shellfish monitoring can also be used to aid source tracking from terrestrial felids, the only known source of *T. gondii* oocysts (Miller et al. 2008; Shapiro et al. 2015).

Outside of New Zealand, prevalence of *T. gondii* in marine shellfish collected throughout the year has been found to range between 0.07% and 9.4% (Aksoy et al. 2014; Esmerini et al. 2010; Marquis et al. 2015; Miller et al. 2008; Putignani et al. 2011; Ribeiro et al. 2015; Shapiro et al. 2015). Seasonal differences have been observed in California, USA, where rainy season sampling appears to be associated with high *T. gondii* prevalence in mussels (Shapiro et al. 2015), reaching 46.3% in a small recent study (Staggs et al. 2015). Exposure to freshwater outflow has also been observed as a significant risk factor for *T. gondii* contamination of mussels (Shapiro et al. 2015). In general, factors that affect pathogen loading and transport from land to sea are likely to be associated with nearshore *T. gondii* pollution. These factors include precipitation patterns, watershed size, land cover/use, domestic and wild cat density within a watershed (VanWormer et al., 2016; Vanwormer et al., 2013).

In New Zealand, previous research has shown that the green-lipped mussel (*Perna canaliculus*), common around the coastline, may be a good sentinel species for assessing marine contamination with *T. gondii* (Coupe et al. 2018; Chapter 6). A total of 13 out of 104 (12.5%; 95% CI 7.09 – 20.8) commercially-sourced green-lipped mussels from New Zealand were found to be positive for *T. gondii* DNA, with a significantly higher prevalence observed in mussels collected in the summer than the winter (Coupe et al. 2018; Chapter 6). Building on these results, the aim of the present study was to investigate *T. gondii* in green-lipped mussels collected from field sites located in key Maui dolphin habitats. The specific objectives were: 1) to use the best-performing validated molecular technique from Chapter 7 to determine the prevalence of *T.*

gondii in the mussels; 2) use PCR-RFLP analysis to determine the molecular genotype(s) of *T. gondii* present, and; 3) to test the hypothesis that *T. gondii* contamination in mussels is associated with (i) high levels of precipitation (ii) sampling during the summer (iii) larger watershed area (iv) greater companion cat density. By determining the prevalence of contamination in mussels at key locations and risk factors for contamination, the exposure risk for Maui's dolphins can better be understood, enabling rational and targeted management measures.

8.2 Methods

8.2.1 Study design

Mussel sampling began in April 2014 and ended in January 2017. During this period, mussels were collected approximately bi-monthly from two field sites along the West Coast of the North Island of New Zealand, near the towns of Raglan and Port Waikato (see Table 21 and Map 2). Sites were chosen due to their location within Māui dolphin habitats (meaning results would be directly relevant to dolphin risk assessment models) and feasibility of access to mussel beds. Although close in terms of distance (47 km/29 miles), these sites were considered to differ in terms of the amount of surface runoff (watershed area) and density of cats in the catchment area, permitting analysis of these variables as risk factors for coastal contamination with *T. gondii* oocysts.

Site 1 (Raglan; Lat -37.8167, Long 174.8296) was considered to have relatively low cat densities (using human population density in the catchment as a proxy variable) and a low level of surface run-off. Raglan is a small beach town (population 2,736 according to the 2013 Census) and is the main township in the Whaingaroa catchment. The catchment does contain other very small settlements like Te Mata (population of 132), Te Uku, and Waingaro, but no other towns or cities. It is separated from the nearest large city of Hamilton, flanked by ranges of hills, making it a discrete, relatively small (525km^2) and rural catchment (Environment Waikato 2002). Site 2 (Port Waikato; -37.3957, 174.7113) is also a small seaside town

(population 1006 according to the 2013 Census). However, the Waikato River, which runs into the Port Waikato Harbour, has an extremely large catchment. The Waikato River is the longest river in NZ, with a length of 425 km and has the second largest catchment area in the country (Catchment Area 14,456 km^2, Environment Waikato 2008). The Waikato River arises on the snow-covered slopes of Ruapehu in the central North Island at an elevation of 2797 metres above mean sea level (amsl) and flows through the large urban area of Hamilton and other towns. Within the catchment there are about 22,200 km of rivers and streams. Before it enters the Tasman Sea through a narrow channel at Port Waikato, the river passes through a delta (a myriad of small interconnected channels and islands formed by deposition of sediment) and then passes in to Maioro Bay.

For risk factor analysis, sites were to be modeled together using logistic regression analysis including site (watershed size), season, precipitation level preceding sampling, and cat density in the catchment, as risk factors. Climate, hydrological, and demographic data were obtained from the Waikato Regional Council (Environment Waikato 2002, 2008), Stats NZ geographic data service (https://datafinder.stats.govt.nz/), or New Zealand National Institute of Water and Atmosphere database, freely accessed through the CliFlo website (http://cliflo.niwa.co.nz/).

Mussels were collected from natural mussel beds at both sites, in accordance with New Zealand Government Fisheries guidelines (maximum of 50 mussels per person per day; no permit required) (Industries MfP). At each sampling effort, approximately 100 mussels were collected from each site. Mussels were processed on-site within 12 h of collection, as described in Chapter 6. Briefly, outer shell surfaces were washed by hand with tapwater before a notch was filed to allow aseptic aspiration of haemolymph from the posterior adductor muscle using a sterile 22-gauge needle (Shapiro et al. 2015). Haemolymph samples were then frozen at -20°C, kept cool, and transported to Massey University, Palmerston North, New Zealand.

8.2.2 DNA extraction and PCR

8.2.2.1 Initial test batches

Initial test batches were included in the study. These batches included haemolymph samples from mussels collected in April, May, and July 2014 (*n* = 166) from Raglan (*n* = 156, April and May), and Port Waikato (*n* = 10, July). After aspiration, haemolymph was stored at -20°C for no longer than two weeks before DNA extraction and PCR testing. For DNA extraction, haemolymph was thawed at room temperature and 100 µl of haemolymph per mussel was aliquoted for processing, then centrifuged at 20,000 x *g* for 10 mins. The supernatant was removed and the haemocyte cell pellet was resuspended in 100 µl PBS (1X, pH 7.4). DNA was extracted from individual mussel samples using DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany) as per manufacturer's instructions for non-nucleated blood (spin column protocol), incubating the samples at 56°C overnight after addition of Buffer AL and proteinase K, and eluting DNA in 100 µl of Buffer AE. DNA was stored at -20°C until used for molecular analysis. A nested PCR targeting the *dhps* gene of *T. gondii* (Aspinall, Marlee, Hyde, & Sims, 2002) (later validated in Chapters 6 and 7, Coupe et al. 2018, 2019) was used to detect *T. gondii* DNA in the initial test batch mussels. Haemolymph remaining after the initial test batch processing was archived at -80°C along with the subsequent samples collected and was re-used in the full study.

8.2.2.2 Full study

In the full study, haemolymph samples which had been stored at -80°C for 5 – 36 months were thawed at room temperature and 100 µl haemolymph per mussel was aliquoted, pelleted, and suspended in PBS, as above. DNeasy Blood and Tissue kits (Qiagen) were also used for DNA extraction in the full study but the protocol was modified following procedure detailed in Shapiro et al. (2015), to be consistent with overseas studies. Briefly, 180 µl of Buffer ATL was added and samples were subjected to one cycle of freeze-thaw by submersion in liquid nitrogen (4 min), followed by immediate transfer into a boiling water bath (4 min). Approximately 40 µl of proteinase K was then added to each sample and maintained at 56°C overnight for digestion. DNA was eluted in 50 µl 95°C ultrapure water with 10 % Buffer AE. Extraction controls using PBS

(1X, pH 7.4) were included. DNA was stored at -80°C until further PCR analysis. Screening of mussel haemolymph for *T. gondii* in the full study was performed using the PCR assay determined most sensitive for parasite detection in the oocyst spiking experiments carried out in Chapter 7: real-time PCR targeting the 529 bp repetitive element of *T. gondii* (rep529 qPCR).

8.2.3 Troubleshooting process

Due to the discrepancy between prevalence results from initial test batches and the full study, differences between initial test batch methods and those of the full study were closely examined to establish the cause. An overview of method differences is provided in Figure 15. Points of interest included: 1) human error; 2) PCR assay used for *T. gondii* DNA detection; 3) DNA extraction method; and 4) storage time. Human error was ruled out as the cause of the discrepancy because DNA extractions in the full study were carried out by two individuals [A. Coupe and E. Burrows] and no difference in prevalence was observed between samples processed by either individual. Also, both the PCR methods in question had been validated using *T. gondii* oocyst spiking experiments prior to the full study, and the rep529 qPCR was found to be more sensitive than the nPCR *dhps* (Coupe et al. 2019, Chapter 7).

The change in extraction protocol was also ruled out; the updated extraction protocol was employed because current thinking is that freeze-thaw(s) using liquid nitrogen and high temperatures are required to release *T. gondii* sporozoites from within the robust oocyst wall (Hohweyer, 2013; Manore, Harper, Aguilar, Weese, & Shapiro, 2019). However, the yield and purity of DNA extracted in the initial test batches (Extraction 1) was found to be superior to that of DNA extracted in the full study (Extraction 2) (assessed using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific)). To investigate this finding, a subset of mussels with remaining haemolymph and which were *T. gondii* positive in the initial test batches, were processed using the original extraction method (Extraction 1') and screened for *T. gondii* DNA using both the nPCR *dhps* and the rep529 qPCR. Yield and quality of Extraction 1' DNA was no better than from Extraction 2 and the new DNA samples were found to be negative for *T. gondii* DNA, suggesting that

updating the extraction protocol was not the cause of the discrepancy between prevalence results from initial test batches and the full study. In the full study, mussels collected on both 'high'- and 'low'- risk sampling dates were also tested to ensure *T. gondii* presence had not been missed through accidental testing of batches expected to have low/zero prevalence alone. High and low risk sampling dates were determined by calculating and assessing cumulative rainfall (mm) in the 30 days before mussel collection (see Table 21 and Appendix 8). It is thought that high levels of rainfall are a risk factor for marine contamination, particularly following dry periods, as oocysts that have accumulated on land are flushed in land-sea runoff to the coast (VanWormer et al. 2016). The low-risk sampling date chosen in this study was the date/location that had relatively low levels of rainfall in the preceding weeks to month. Conversely, the high-risk sampling date was the date/location that had relatively high levels of rainfall in the preceding weeks to month.

Table 21 Mussel sampling efforts and dates between April 2014 and January 2017 at two sampling sites along the West Coast of the North Island of New Zealand, near the towns of Raglan and Port Waikato. Two sampling dates were chosen as 'high' and 'low' risk dates in terms of rainfall levels in the preceding weeks to month.

Field Site	Sampling Date	High- or Low-Risk	Number of Mussels	
Raglan	11-Apr-14		59	
	13-May-14		97	
	10-Jul-14		97	
	21-Dec-14		110	
	14-Feb-15	Low	101	
	6-Apr-15		101	
	15-Jul-15		102	
	10-Oct-15		97	
	9-Mar-16		100	
	1-Jul-16	High	102	
	21-Jan-17		101	
Port Waikato	11-Jul-14		86	
	12-Jul-14		98	
	1-Oct-14		101	
	13-Jun-15		93	
	9-Oct-15		100	
	10-Mar-16		101	
	2-Jul-16		100	
	20-Jan-17		99	
Total				1844

8.2.3.1 Storage study

Troubleshooting so far led to the hypothesis that long-term storage of haemolymph negatively affects the ability to detect *T. gondii* DNA in samples. A storage study was therefore designed to assess whether there was a significant difference in *T. gondii* prevalence between freshly processed and stored haemolymph samples. Fifty mussels were collected from the Raglan field site in August 2017. For each mussel, haemolymph was extracted as described earlier, vortexed and divided into two aliquots. One aliquot (Aliquot 1) was processed within 72 h and the other (Aliquot 2) was stored at -80°C for 5 months before processing. The same DNA extraction protocol used in the initial test batches was used throughout the storage study, and haemolymph was tested for *T. gondii* DNA using the nPCR *dhps* (Table 22). Controls consisting of oocyst-spiked haemolymph were also included in the storage study. Aliquot 1 haemolymph samples that tested negative for *T. gondii* DNA using the nPCR *dhps* were pooled and divided into two sets of 9 x 100 µl aliquots and each aliquot was spiked with 500 *T. gondii* oocysts (using the serial dilution prepared in Chapter 7). DNA was extracted from one set of control aliquots immediately; the other set was stored at -80°C for 5 months before processing. A McNemar's test was used to test the difference between the paired proportions. Significance was indicated when $P \leq 0.05$. Statistical analyses were performed in R version 3.4.4 (R Core Team 2017) using base and binom (Dorai-Raj 2014) packages.

Figure 15 Overview of method differences between initial test batches and full study for processing and screening mussel haemolymph samples for *T. gondii* DNA. **1** initial test batches; **2** full study

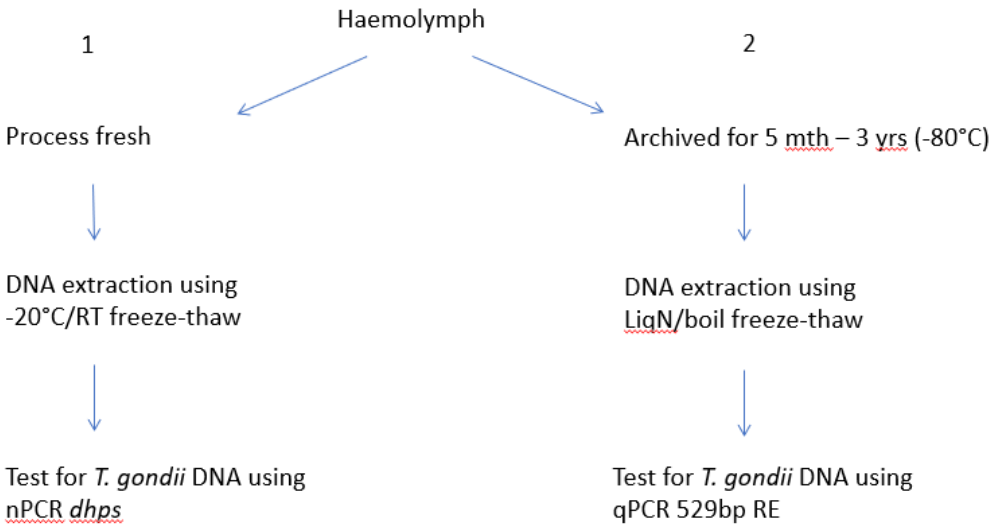

Table 22 Summary of results from the storage study in which 50 new green-lipped mussels were collected from Raglan and tested for *T. gondii* DNA using the same methods as employed in the initial batches. Mussel haemolymph samples and controls were tested fresh and after storage at -80°C to determine the effect of long-term storage on parasite detection.

	Haemolymph storage time	**Extraction method**	**PCR assay**	**Proportion positive for *T. gondii* DNA (%, 95 % CI)**
50 haemolymph samples	<2 weeks	Extract -20°C/RT	nPCR *dhps*	1/50 (2, 0.35 – 10.5)
	5 months	Extract -20°C/RT	nPCR *dhps*	0/50 (0, 0 – 7.1)
Controls: haemolymph spiked with 500 oocysts (*n* = 9)	<2 weeks	Extract -20°C/RT	nPCR *dhps*	4/9 (44, 18.9 – 73.3)
	5 months	Extract -20°C/RT	nPCR *dhps*	3/9 (33, 12.1 – 64.6)

8.3 Results

A total of 166 mussels was included in the initial test batches; 156 were collected in Raglan (11.04.2014, 13.05.2014) and ten were collected in Port Waikato (12.07.2014). Overall prevalence of *T. gondii* DNA was determined to be 19.3 % (95 % CI: 14.0 – 25.9) using the nPCR *dhps*. A total of 1844 mussels was processed for the full study and of these 246 were tested for *T. gondii* DNA using the rep529 qPCR, including 43 mussels which had haemolymph remaining after processing in the initial test batches (collected in Raglan 11.04.2014). Mussels collected on high- and low-risk sampling dates were included in the full study testing (see Table 21 and Appendix 8). *Toxoplasma gondii* DNA was not detected in any of the haemolymph samples tested in the full study.

In the storage study, using the nPCR *dhps*, *T. gondii* DNA was detected in 1/50 (2%, 95% CI: 0.35 – 10.5) mussels that were processed within 72 h and in 4/9 (44.4 %, 95% CI: 18.9 – 73.3) of the corresponding oocyst-spiked controls. After haemolymph was stored for 5 months at -80°C, *T. gondii* DNA was detected in 0/50 (0%, 95% CI: 0 – 7.1) mussels and in 3/9 (33.3%, 95% CI: 12.1 – 64.6) oocyst-spiked controls (Table 22). The proportion positive was lower in the stored groups relative to the groups processed within 72 h, but the differences were not statistically significant.

8.4 Discussion

The aim of the present study was to investigate *T. gondii* in wild green-lipped mussels collected from field sites located in key Māui dolphin habitat, as bio-sentinels of marine contamination. In total, 1844 mussels were collected from Raglan and Port Waikato between April 2014 and January 2017. A relatively high *T. gondii* prevalence in the full study was expected: in the initial stages of the study 32/166 (19.3 %) of mussel haemolymph samples tested were positive for *T. gondii* DNA. Results of the subsequent supermarket mussel study (Coupe et al. 2018; Chapter 6) also supported this expectation, with a *T. gondii* prevalence of 12.5 % (13/104) in commercially sourced green-lipped mussels in New Zealand, indicating that this parasite is likely to be widespread in New Zealand coastal waters. It was further hypothesised that *T. gondii*

prevalence would be significantly higher in mussels sampled in summer relative to winter (Coupe et al. 2018; Chapter 6) and when the sampling date occurred after rain events (Shapiro et al., 2015; VanWormer et al. 2013, 2016). In the full study, however, none of the haemolymph samples tested were positive for *T. gondii* DNA, even those collected on a theoretically high-risk date in terms of precipitation (01/07/2016, Raglan), and despite the use of the rep529 qPCR, which was determined to be a more sensitive assay for *T. gondii* detection relative to the nPCR *dhps* (Coupe et al. 2019, Chapter 7). A troubleshooting process was therefore employed to explain the discrepancy in results between initial test batches and those of the full study, discussed in detail in the Methods section. Briefly, human error, the use of an alternative DNA extraction method and PCR assay for *T. gondii* DNA detection, were systematically ruled out as the cause of the discrepancy. A storage study was ultimately implemented to test the hypothesis that long-term storage negatively affects *T. gondii* DNA detection in mussel haemolymph.

In the storage study, the proportion of mussel haemolymph samples that tested positive for *T. gondii* DNA was found to be lower in stored samples compared with samples processed within 72 h, although the differences were not statistically significant. In theory, storage at -80°C should preserve DNA in tissue samples (Shabihkhani et al., 2014) but results of the present study suggest that this may not be the case for green-lipped mussel haemolymph. Other shellfish studies using molecular methods to detect *T. gondii* have reported storage of haemolymph and other tissues at -80°C or -20°C before processing (Arkush et al. 2003; Esmerini et al. 2010; Kerambrun et al. 2016; Miller et al. 2008; Ribeiro et al. 2015; Staggs et al. 2015), but have not provided details on storage time, so further research is required to confirm whether long-term storage of haemolymph at -80°C (or -20°C) negatively impacts the quality and/or yield of extractable DNA. This is of particular importance for studies investigating *T. gondii* in shellfish as only low numbers of oocysts are expected to be found in mussel tissues (< 100 per mussel, Hohweyer 2013), meaning only low concentrations of *T. gondii* DNA are expected in total DNA extracted from mussel haemolymph.

Despite the absence of *T. gondii* DNA in haemolymph samples tested in the full study, 2 % (1/50) of haemolymph samples processed within 72h of collection and tested in the storage study, were found to be

positive. Although a lower prevalence than expected (perhaps due to the small sample size or inclusion of a single sampling date), this proportion is within the *T. gondii* prevalence range for marine shellfish collected throughout the year outside of New Zealand (0.09 % to 9.4 %, Aksoy et al. 2014; Esmerini et al. 2010; Marquis et al. 2015; Miller et al. 2008; Putignani et al. 2011; Ribeiro et al. 2015; Shapiro et al. 2015). This result supports previous findings that *T. gondii* oocysts are reaching coastal waters in sufficient quantities to be detected in mussels grown in New Zealand (Coupe et al. 2018, Chapter 6). The result also confirms that *T. gondii* is still present in the habitat of the critically endangered Maui's dolphin subspecies and therefore remains a pathogen of concern for the Hector's dolphin. In addition to their use as sentinels of marine contamination with *T. gondii*, green-lipped mussels are harvested recreationally for human consumption in New Zealand. Detection of *T. gondii* in the storage study mussels further indicates that wild-harvested mussels as well commercially-grown mussels (Coupe et al. 2018, Chapter 6) are a potential health risk for consumers.

Until better techniques become available for direct testing of seawater, the use of shellfish as bio-sentinels will remain an important tool by which to determine prevalence and genotypes of *T. gondii* in the marine environment, plus risk factors for marine contamination. As many shellfish species are consumed by humans, surveillance for *T. gondii* is also important for public health. It is therefore valuable to provide recommendations for continued investigation of *T. gondii* in green-lipped mussels in New Zealand. In particular, DNA should be extracted from green-lipped mussel haemolymph within 72 h of haemolymph collection, until more in-depth studies are carried out to determine optimal haemolymph storage conditions. As only low numbers of *T. gondii* oocysts are expected per mussel, a further recommendation is to centrifuge all haemolymph aspirated from each individual mussel, retaining 100 µl of the haemocyte cell pellet for DNA extraction (Shapiro et al. 2015), rather than centrifuging 100 µl haemolymph per mussel (this study), in order to maximise the chances of detecting *T. gondii* DNA, if present.

With respect to surveillance study design, sampling sites and times should be chosen in order to assess the distribution of the parasite in New Zealand waters, risk factors for marine contamination, and exposure risk

for Hector's dolphins. This will entail selecting geographical regions for field sampling within Hector's dolphin habitat, particularly where *T. gondii* exposure or fatal toxoplasmosis has been documented (Roe et al. 2013), as in this study. Regions outside Hector's dolphin habitat are also of interest in order to gain further information about the susceptibility of Hector's dolphins to the disease (see Chapter 2). To evaluate cat density and amount of surface runoff as risk factors for *T. gondii* contamination of wild mussels, sampling regions should differ in terms of the density of cats in the catchment area, as well as the watershed size. Within each region, sampling could further be structured to evaluate whether *T. gondii* contamination is associated with proximity to freshwater runoff (Shapiro et al. 2015). To do so, each region should include at least two sampling sites – one proximal and the second distant (at least 5 km) to a freshwater source (Shapiro et al. 2015). To assess precipitation levels and season as risk factors, mussels should be collected bi-monthly for at least two years.

Map 2 A Map of New Zealand showing the Waikato region (grey shading) and range of the Māui dolphin subspecies of Hector's dolphin (*Cephalorhyncus hectori maui*) (blue shading) **B** Map of the Waikato region showing the sampling sites at Port Waikato and Raglan and their associated catchments. The catchment for Port Waikato (grey shading) is the second largest in New Zealand and includes the Waikato river that flows through Lake Taupo as well as the Waipa river which joins the Waikato. The rivers flow through the large urban city of Hamilton. The catchment for Raglan is relatively small, rural, and discrete (green/grey shading), separated from Hamilton by ranges of hills

8.5 References

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9 General Discussion

9.1 Introduction

The over-arching aim of this thesis was to investigate land-sea transmission of the protozoan parasite, *Toxoplasma gondii*, in New Zealand. Already accepted as a serious problem for ovine livestock in New Zealand for many years (Hartley & Marshall, 1957), *T. gondii* has more recently been identified in endemic marine mammals (Roe et al., 2013, 2017; Michael et al., 2016), suggesting that environmentally resistant *T. gondii* oocysts, shed only in cat faeces, are reaching marine ecosystems (Miller et al., 2002; VanWormer et al., 2016; Shapiro et al., 2019a). An unusually high prevalence of *T. gondii* infection and fatal toxoplasmosis in a case series of stranded and bycaught Hector's dolphins led Roe et al. (2013) to question why this species appears to be particularly susceptible to toxoplasmosis. Moreover, a certain genotype of *T. gondii*, namely variant Type II (ToxoDB #3), was identified as the cause of fatal disseminated toxoplasmosis in Hector's dolphins (Roe et al., 2013) as well as native bird species (Howe et al., 2014), leading Roe et al. (2017) to hypothesise that this genotype is either particularly pathogenic or is widespread in the New Zealand environment. These findings and hypotheses formed the basis of this research. To investigate land-sea transmission of *T. gondii* in New Zealand and to provide evidence for or against these hypotheses required examination of *T. gondii* in other New Zealand cetaceans, in cats, as the definitive host of the parasite and source of oocysts, and in the green-lipped mussel, as a biosentinel for *T. gondii* contamination of the marine environment.

A review of the relevant literature was provided in Chapter 1. This offered insights into how to study land-sea transmission in New Zealand and highlighted a number of gaps in the published research. Particularly, the review underscored that little published information about environmental transmission and epidemiology of *T. gondii* in the marine environment in the New Zealand was available. *Toxoplasma gondii* infection had not systematically been investigated in any other cetacean species in the country, nor had the prevalence of *T. gondii* in coastal waters been determined. Despite the long history of *T. gondii* affecting

sheep in the country, no data was available estimating the oocyst burden in New Zealand, and only anecdotal evidence of *T. gondii* infections in New Zealand cats could be uncovered (Thompson, 1999).

The literature revealed that animal infections with *T. gondii* generally follow a similar path to those in humans, with widespread exposure to the parasite documented in many species across the globe, but clinical toxoplasmosis generally limited to at-risk populations (Dubey, 2016). However, *T. gondii* appears to be particularly pathogenic for some marine mammals, notably Southern sea otters (Kreuder et al., 2003), and perhaps Hector's dolphins (Roe et al., 2013). Long-term studies on the Southern sea otter were thoroughly discussed as a benchmark by which to study toxoplasmosis in other marine mammals. This body of work highlighted that the epidemiology of *T. gondii* infection in otters and other marine mammals is complex and that reasons for susceptibility and disease are not yet fully understood. Potential risk factors for otter exposure determined to date, however, include age, diet, proximity to freshwater outflow, domestic cat density, human population density, anthropogenic development, and land use (Miller et al., 2002; Burgess et al., 2013, 2018; VanWormer et al., 2016; Shapiro et al., 2019a; Tinker et al., 2019). Significant associations between otter mortality and *T. gondii* genotype, season, and year of sampling, have also recently been identified (Shapiro et al., 2019b). Importantly, the review highlighted that these results relied on many years of in-depth live-sampling, tracking, and complete necropsy data, making analogous studies on the Hector's dolphin in New Zealand presently infeasible, due to logistical and funding constraints, as well as the ecology of the Hector's dolphin. As such, alternative means to investigate the role of toxoplasmosis in the population decline, or failure to recover, of the Hector's dolphin were explored.

The literature suggested that the examination of sympatric species can be informative. For example, California sea lions have ranges that overlap those of the Southern sea otter, but the former species were found to have a significantly lower *T. gondii* antibody prevalence, suggesting that different feeding ecologies and migratory patterns can affect exposure to infectious oocysts within the same ecosystem (Carlson-Bremer et al., 2015). At the onset of the project, detailed investigations of *T. gondii* infection had not been conducted in any other cetacean species except the Hector's dolphin in New Zealand. This was

considered an important avenue to help establish whether the Hector's dolphin is unusually susceptible to *T. gondii* infection amongst New Zealand cetaceans, which was followed in Chapter 2.

Research conducted in California also stressed the importance of establishing the extent of, and risk factors associated with, coastal contamination with *T. gondii*, as a means to better understand the distribution of *T. gondii* oocysts in marine habitats and the exposure risk for marine mammals (Shapiro et al., 2015). The best current option for surveillance in coastal waters was determined to be the use of bivalves as biosentinels for *T. gondii*. Interestingly, although oocysts have been shown to survive for months to years in saltwater (Lindsay & Dubey, 2009), studies have failed to molecularly confirm the presence of *T. gondii* oocysts in seawater samples thus far (Shapiro et al., 2019a). Research has therefore been carried out to develop filter-feeding shellfish as biosentinels; experimentally it has been shown that shellfish can take up and accumulate *T. gondii* oocysts, and a number of naturally exposed shellfish species across the globe have now been found to be positive for *T. gondii* DNA or RNA (e.g. Esmerini et al., 2010; Putignani et al., 2011; Aksoy et al., 2014; Zhang et al., 2014; Shapiro et al., 2015). Green-lipped mussels were noted to be endemic to New Zealand and abundant in near-shore waters throughout the country. As such, methods were developed for their use in *T. gondii* surveillance in New Zealand nearshore waters, in efforts to better understand toxoplasmosis risks for the Hector's dolphin (Chapters 6, 7, and 8).

Research has shown that oocysts of *T. gondii* cannot replicate outside of the feline definitive host. Therefore, knowledge of feline infections and shedding prevalence has proved essential to estimate the environmental oocyst burden and to determine feline-related factors that may impact marine mammal exposure and mortality from toxoplasmosis (Dabritz et al., 2007; Dabritz & Conrad, 2010; VanWormer et al., 2013). Notably, studies in California and elsewhere found an outdoor lifestyle and diet made up of many small prey items to be associated with higher levels of feline infection and shedding (VanWormer et al., 2013). Furthermore, cats of all of ages were determined to be a potential source of oocysts due to the possibility of re-shedding after first infection (Shapiro et al., 2019a). Recent sea otter related studies also identified feline shedding prevalence and cat densities as important predictors of sea otter exposure

(VanWormer et al., 2016; Burgess et al., 2018), making studies of *T. gondii* in cats particularly important for risk assessment and control of *T. gondii* transmission. In California, for example, research has had an impact on local policy; California is the only state in the USA that requires cat litter manufacturers to provide statements to encourage the disposal of cat faeces with household waste and to discourage consumers from flushing cat faeces in toilets or disposing of them in drains. Owners are also encouraged to keep their cats indoors. Considering New Zealand, studies have revealed that a very high proportion of pet cats are allowed outdoors (Farnworth et al., 2010; Hall et al., 2016), where they can hunt and deposit faeces. Very little, however, has been published about *T. gondii* in New Zealand cats, with no baseline data available on the number of cats infected with *T. gondii* or shedding oocysts, a gap that Chapters 3, 4, and 5 aimed to fill.

The literature also revealed that genotyping of *T. gondii* isolates is essential, both to study transmission routes of the parasite and to investigate marine mammal susceptibility to toxoplasmosis. With respect to the Southern sea otter, linkages between *T. gondii* genotype and increased otter susceptibility were first suspected back in 2004 (Miller et al., 2004), and a recent in-depth molecular analysis has now found a statistically significant association between *T. gondii* genotype and mortality outcomes (Shapiro et al., 2019b). Moreover, this, and earlier work showed that the same virulent strains discovered in sea otters were also found in felids living nearby (VanWormer et al., 2014). Results suggested that knowledge of the distribution of *T. gondii* genotypes in cats is required to identify sources of infection for intermediate hosts, such as the sea otter, as well as to identify high-risk cat populations for management purposes. Further, the discovery of an identical and atypical *T. gondii* allele in mussels, as in the sea otter and neighbouring felids, strongly indicated that land-to-sea transmission of novel *T. gondii* strains had occurred through overland run-off containing cat faeces. In New Zealand, the findings of Roe et al. (2013) suggested that representatives of the variant Type II *T. gondii* lineage may be particularly pathogenic for the Hector's dolphin. However, whether these strains were particularly prevalent in New Zealand, whether they could

be traced back to specific cat populations or habitats, or could be located in the marine environment, had not been investigated, a gap which this thesis attempted to address throughout.

9.2 Land-sea transmission of *T. gondii* in New Zealand

Figure 16 depicts the likely course of *T. gondii* oocysts from land to sea in New Zealand as well as routes of *T. gondii* transmission for the Hector's dolphin and other species in the country. This thesis attempted to evaluate links in the land-sea transmission chain to better understand *T. gondii* in the marine environment in New Zealand. The following sections discuss the main findings and implications of this evaluation.

9.2.1 *Toxoplasma gondii* is widespread in New Zealand cats

Freshwater runoff contaminated with cat faeces is considered to be the source of sea otter infection in the USA (Miller et al., 2002, 2004, 2008) and this is likely to be the case in New Zealand. It is therefore important to assess the terrestrial oocyst burden as this will impact marine mammal exposure risk. As such, Chapters 3 and 4 aimed to determine the prevalence of *T. gondii* infection and oocyst shedding in New Zealand cats. Specifically, Chapter 3 aimed to provide a preliminary estimate of true seroprevalence in naturally exposed companion cats in the country. If a cat has antibodies to *T. gondii,* this indicates exposure to the parasite at some time during its life, previous oocyst shedding, and the possibility of current repeat shedding (Dubey & Frenkel, 1972; Dubey & Thulliez, 1989; Davis & Dubey, 1995; Dubey et al., 1995). Chapter 4 investigated oocyst shedding in New Zealand cats through direct faecal examination, as although the seroprevalence study was essential to estimate the extent of *T. gondii* infection, the tests used in Chapter 3 could not confirm precisely when the cat was shedding oocysts. Oocyst shedding prevalence is generally found to be low in cat populations, typically <2 % where the estimate is based on faecal flotation and light microscopy (e.g. Dabritz et al., 2007b; Schares et al., 2016). To maximise the chances of finding oocysts, this study therefore targeted feral and stray domestic cats, rather than owned cats, as the former are more likely to be actively infected through ingestion of prey (Wilson, 2004; Gillies &

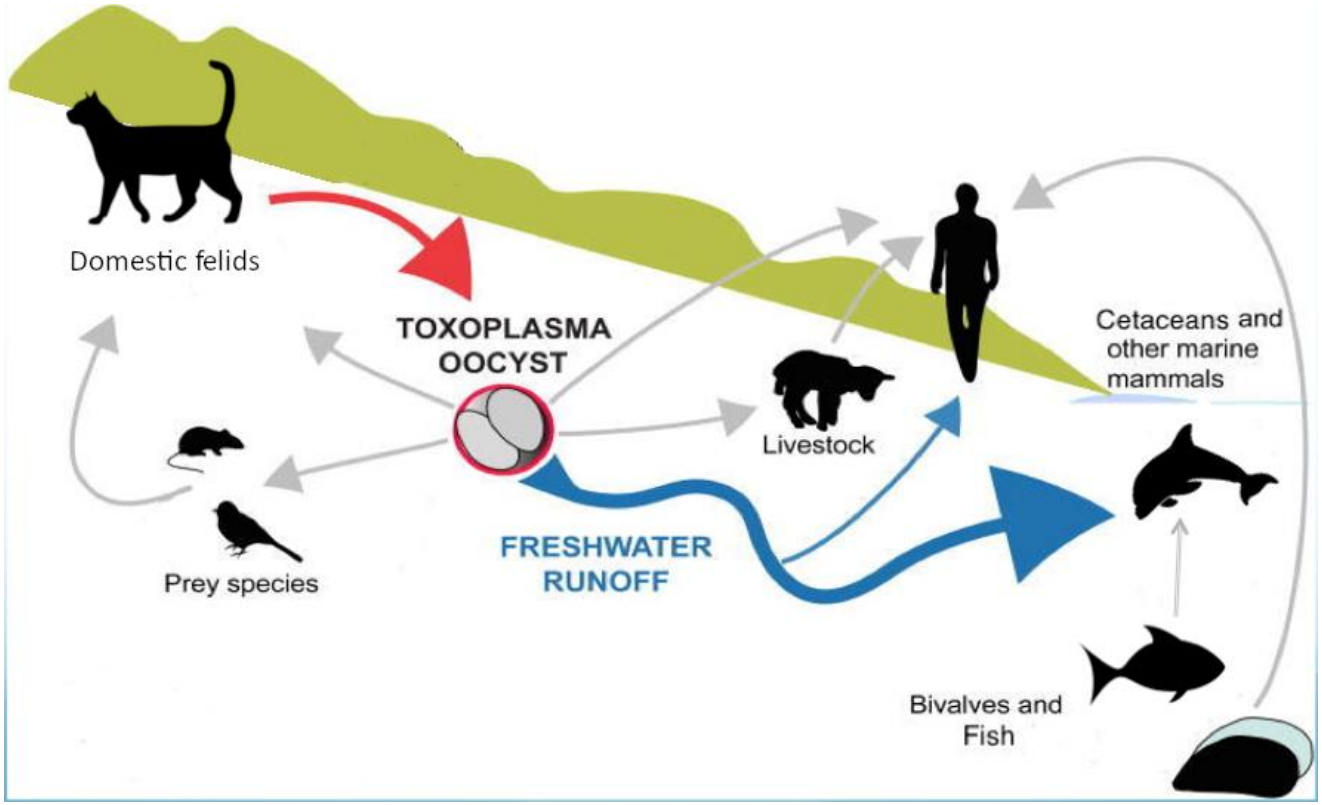
Fitzgerald, 2005). As a potentially more sensitive means of detecting oocyst shedding, the prevalence of *T. gondii* DNA in whole faeces was also estimated.

True *T. gondii* seroprevalence amongst owned cats was found to be 61% (95% CrI: 48 – 76), which was relatively high compared to the worldwide seroprevalence estimate of 30 – 40% (Elmore et al., 2010; Dubey, 2016). In contrast, the proportion of feral and stray cats shedding *T. gondii* oocysts in their faeces was estimated to be 1.6 %, which was within expected limits considering global estimates (e.g., Miró et al., 2004; Pena et al., 2006; Dabritz et al., 2007; Karatepe et al., 2008; Schares et al., 2008, 2016; Herrmann et al., 2010; Mancianti et al., 2010; Berger-Schoch et al., 2011; Jokelainen et al., 2012). Nevertheless, using this new knowledge, the annual density of oocyst loading in New Zealand could be calculated, and it was determined that between 51 and 62 trillion oocysts are shed into the environment in a year by owned, stray, and feral cats together, assuming there are between 0.4 – 0.7 million feral and stray and 3 million owned cats in the country and that the shedding prevalence in owned cats approximates that in ferals and strays. It should be noted that there is high uncertainty surrounding feral and stray population densities which may affect this estimate, but still an enormous amounts of oocysts are expected to be deposited annually, posing a risk for susceptible intermediate hosts. Importantly, both seropositive cats and cats actively shedding oocysts were found throughout New Zealand. In the sero-survey, although sample sizes were small, results suggested that seroprevalence was consistent across all eight regions included in the study. Although it was not possible to include region as a risk factor in the shedding prevalence study, cats actively shedding *T. gondii* oocysts were sampled from both the North and South Island of New Zealand. Furthermore, cats positive for *T. gondii* DNA in whole faeces were sampled from each of the ten regions included in the study.

Taken as a whole, results of Chapters 3 and 4 established that *T. gondii* infection is widespread in owned, stray and feral cats in New Zealand, and implied that *T. gondii* infected prey is prevalent across New

Zealand. Results also demonstrated the potential for a high level of environmental contamination with *T. gondii* oocysts on both islands of New Zealand, including regions that border Hector's dolphin habitat, where oocysts may feasibly be transported to the ocean in land-sea run-off. The large, estimated oocyst burden suggests substantial exposure risks for nearshore marine mammals, especially Hector's dolphins, which are known to forage in shallow, estuarine waters (Rayment et al., 2010; Dawson et al., 2013). Cats confirmed to be actively shedding oocysts by faecal flotation and PCR were sampled from Canterbury (bordering Hector's dolphin habitat), Manawatu, and Auckland (Great Barrier Island). Finding cats actively shedding oocysts on Great Barrier Island also has important implications for other New Zealand wildlife. Although the island lies outside the Hector's dolphin range, it includes the Aotea Conservation Park and is home to rare avifauna which may also be susceptible to toxoplasmosis (Howe et al., 2014). Using the whole faeces PCR results it was also possible to get a preliminary look at potential risk factors for cat exposure to *T. gondii* and oocyst shedding. Results suggest that feral cats are more likely to come into contact with *T. gondii* and potentially shed oocysts than strays in New Zealand. This could have important implications for control measures, implying that targeted control of feral cats may be especially important in the country. However, further research is needed to confirm this, and to determine the extent of active shedding in pet cats in the country, which make up the largest group of cats in New Zealand, with over 1.5 cats owned per household (New Zealand Animal Council 2016). As domestic cat numbers are closely linked to the human population size, and as New Zealanders own more pets per household than anywhere else in the world except for the United States (New Zealand Animal Council 2016), the burden of oocysts in the country is not likely to be reduced any time soon. Moreover, increasing coastal development occurring in New Zealand (Collins & Kearns, 2010), may also facilitate transport of these oocysts into the ocean with an increase in impervious surfaces that facilitate land-sea runoff, as well as a reduction in oocyst-sequestering estuarine wetlands, a concern echoed in California (Shapiro et al., 2010; VanWormer et al., 2016).

Figure 16 Resilient, free-living *Toxoplasma gondii* oocysts, which can be transported in freshwater runoff (blue arrow), likely play a significant role in environmental transmission of *T. gondii* in terrestrial and marine systems. In New Zealand, domestic felids are the only known source (red arrow) of *T. gondii* oocysts. Light gray arrows indicate possible routes of *T. gondii* transmission by exposure directly to oocysts or indirectly through food sources. Adapted from VanWormer et al (2013) with permission.



9.2.2 *T. gondii* oocysts may be prevalent in New Zealand coastal waters

The use of filter-feeding, green-lipped mussels as biosentinels of *T. gondii* in New Zealand coastal waters was investigated in Chapters 6 – 8, providing evidence to suggest that oocysts are widespread in nearshore waters in the country, as on land. Specifically, Chapter 6 (Coupe et al., 2018) aimed to assess the prevalence of *T. gondii* in commercially sourced, green-lipped mussels in New Zealand, using optimised molecular methods. A nested PCR targeting the *dhps* gene of *T. gondii*, previously used to test Hector's dolphins (Roe et al., 2013), was validated for detection of *T. gondii* DNA in shellfish and applied to 104 mussel haemolymph samples. Thirteen mussels were found to be positive for *T. gondii* DNA with an estimated true prevalence of 16.4 %, using Bayesian statistics to take into account test sensitivity and specificity. This result was relatively high compared to overseas studies that also tested marine shellfish throughout the year (0.07 % – 9.4 %) (Miller et al., 2008; Aksoy et al., 2014), perhaps a reflection of the large burden of *T. gondii* oocysts estimated to contaminate the terrestrial environment in New Zealand each year (Chapter 4). Mussels in this study originated from ocean farms so prevalence should reflect marine contamination with *T. gondii*. As such, findings suggest a substantial exposure risk for marine mammals inhabiting the nearshore environment in New Zealand, such as the Hector's dolphin.

Nevertheless, findings of Chapter 8 should also be taken into consideration. Chapter 8 aimed to determine the prevalence of *T. gondii* in green-lipped mussels, wild-harvested at key locations in Māui dolphin habitat, as well as risk factors for their contamination. The success of the study in Chapter 6 was taken as evidence that green-lipped mussels would be good sentinels for *T. gondii* in field studies. Surprisingly (devastatingly!), none of the mussels tested in Chapter 8 were found to be positive for *T. gondii* DNA, despite using the best-performing molecular technique, as determined in Chapter 7 (Coupe et al., 2019), and extensive troubleshooting (discussed later). It is possible that the high *T. gondii* prevalence documented in the commercially sourced mussels in Chapter 6 resulted from cross-transmission of oocysts between mussels in holding tanks, rather than representing true levels of coastal contamination. However, it is more likely that the lack of *T. gondii* detection in the field study of Chapter 8 was due to sample

preservation problems, discussed in more detail below. Briefly, prevalence of *T. gondii* DNA in initial test batches of wild-harvested mussels was determined to be 19.3 % (95 % CI: 14.0 – 25.9) using the nPCR *dhps*, supporting the findings of Chapter 6. As these haemolymph samples had been tested while fresh, a storage study was also carried out in Chapter 8 and, although the differences were not statistically significant, the proportion of positive samples was found to be lower in the stored groups (0 %) relative to the groups processed within 72 h (2 %). Overall, the storage study showed that *T. gondii* was still present in wild green-lipped mussels, albeit at lower prevalence (2 %; 95% CI: 0.35 – 10.5). This is meaningful because it supports previous findings that *T. gondii* oocysts are reaching coastal waters in sufficient quantities to be detected in mussels grown in New Zealand. Moreover, the results also confirmed that *T. gondii* is still present in the habitat of the critically endangered Māui dolphin subspecies and therefore remains a pathogen of concern for the Hector's dolphin.

Although *T. gondii* DNA and RNA has been detected in field-sampled shellfish from around the world, as far as the author is aware, no study has yet been able prove that the shellfish were contaminated with infectious oocysts. This is necessary to definitively establish the marine transmission pathway. At present, the gold standard test for determining oocyst infectivity is considered to be the bioassay, followed by *in vivo* cell culture (Rousseau et al., 2019). However, these assays were not available for this thesis. As oocysts must sporulate outside the definitive host to become infectious, Chapter 6 also validated a reverse-transcriptase PCR, which successfully detected a sporozoite-specific marker (SporoSAG) (Radke et al., 2004) in four of the thirteen positive study mussels. Importantly, this represents the first time that sporulated *T. gondii* oocysts have been confirmed present in shellfish worldwide, although unfortunately this assay cannot explicitly demonstrate that the oocysts were infectious at the exact time of testing (Villegas et al., 2010; Ware et al., 2010).

The investigation of *T. gondii* in biosentinels like the green-lipped mussel can also shed light on risk factors for coastal contamination with the parasite (Shapiro et al., 2015), so presumably for Hector's dolphin

exposure. Interestingly, in Chapter 6, the presence of *T. gondii* in mussels was found to be highest in the summer, and significantly higher during the summer (December, January, February) compared with the winter months (June, July, August) (P = 0.003). Whether or not this means the risk of *T. gondii* exposure for the Hector's dolphin is higher in the summer months remains unclear, however, and is intriguing considering that all fatal cases of toxoplasmosis in Hector's dolphins were documented in individuals recovered in spring (September, October, November) (Roe et al 2013; Roberts et al 2019a). Considering the wider literature, it seems difficult to draw conclusions about the timing of *T. gondii* exposure and subsequent marine mammal mortality. Notably, Shapiro et al. (2012) found no temporal association between overland runoff indicators and sea otter mortality from toxoplasmosis in California. This was attributed to a chronic onset of disease in the sea otter, whereby otters subclinically infected with *T. gondii* later develop clinical signs and die from parasite recrudescence, similar to the pathophysiology in humans. It is possible that the pathophysiology of toxoplasmosis in the Hector's dolphin is equally complex.

9.2.3 Are representatives of variant Type II *T. gondii* widespread in New Zealand or particularly pathogenic for New Zealand wildlife?

Genotypes of *T. gondii* were investigated along the land-sea transmission chain throughout this thesis in an effort to determine whether variant Type II *T. gondii* (ToxoDB #3) strains are widespread in the country, or whether they are particularly pathogenic for the Hector's dolphin and other New Zealand wildlife. This was considered a priority because fatal cases of toxoplasmosis in Hector's dolphins and neuromuscular toxoplasmosis in a New Zealand sea lion were attributed to variant Type II (ToxoDB #3), or variant Type II-like *T. gondii* strains (Roe et al., 2013, 2017). Likewise, fatal toxoplasmosis in endemic wild birds was linked to variant Type II *T. gondii* (Howe et al., 2014).

Direct detection of oocysts in feral and stray cat faeces was carried out in Chapter 4 in order to characterise *T. gondii* genotypes shed by these New Zealand cat populations, to investigate the prevalence of variant *T. gondii* Type II representatives on land, and determine the source of infectious oocysts for the Hector's

dolphin. Three cats were found to be shedding oocysts when sampled, and partial genotyping was carried out on two isolates from feral cats, revealing the presence of two unique recombinant strains. Due to the small sample size, it was not possible to rule out the presence of variant Type II *T. gondii* in the study populations. It was speculated, however, that there may be domestic and sylvatic *T. gondii* cycles co-existing in New Zealand as seen in coastal California, with archetypal genotypes, such as variant Type II type prevalent in owned cats and developed areas, and recombinant or atypical genotypes prevalent in feral cats and undeveloped areas (VanWormer et al., 2014). This would implicate pet cats as the primary source of oocysts for Hector's dolphin. To confirm this may prove difficult as well as controversial, however, as it would require knowledge of genotypes circulating in companion cats, as well as stray and feral cats, gathered in a large-scale cross-sectional survey targeting watersheds adjacent to Hector's dolphin habitat.

Through examination of fatal cases of feline toxoplasmosis in New Zealand, it could be determined whether the same variant Type II *T. gondii* identified in fatal cases of toxoplasmosis in Hector's dolphins and other wildlife was also associated with fatal infections in cats. If so, the study would provide evidence to support the hypothesis that variant Type II *T. gondii* is widespread in the New Zealand environment, and would suggest that this strain is particularly pathogenic for New Zealand animals in general. This was attempted in Chapter 5. Specifically, partial genotypes were obtained for two owned cats. Genotyping revealed the presence of a unique atypical genotype in one cat, which was unusual due to the presence of a Type X allele at the B1 locus, making this the first study to report a Type X-like strain outside of the USA. Genotyping was only successful at four of the eight attempted loci for the other cat, making it difficult to draw conclusions on the infecting strain, however. Although the presence of variant Type II *T. gondii* could not be ruled out in the study, it appears that unique recombinant and atypical genotypes are shed by New Zealand cats and can cause disease in these animals. Results of Chapters 3 – 5 appear to support the hypothesis that variant Type II *T. gondii* is especially pathogenic for certain endemic wildlife species, although further research is needed to confirm this.

Unfortunately, no further information about *T. gondii* genotypes in the marine environment in New Zealand was gleaned in this thesis. No dolphin samples in Chapter 2 were found to be positive for *T. gondii* and, although *T. gondii* was detected in green-lipped mussels (Chapters 6 and 8), genotyping was not successful. This is likely due to low concentrations of parasite DNA in the mussel samples and PCR methods lacking the necessary sensitivity to reliably detect low copy numbers of typing loci in shellfish tissues (Esmerini et al., 2010; Shapiro et al., 2015; Staggs et al., 2015; Chapter 7).

Overall, this thesis provided evidence to further the hypothesis that variant Type II *T. gondii* is especially pathogenic for certain endemic wildlife species in New Zealand, including the Hector's dolphin. However, the evidence was not sufficient to discount the possibility that variant Type II *T. gondii* is also prevalent along the land-sea transmission chain in the country. Considering the wider literature, research has shown variant Type II *T. gondii* to be one of the most common genotypes worldwide, despite regional specificities and a higher prevalence of atypical genotypes in some locales (Shwab et al., 2014; Jiang et al., 2018). Furthermore, it is likely that *T. gondii* was established in New Zealand relatively recently with the introduction of the domestic cat from Europe. It is therefore possible that the strains in New Zealand reflect those in Europe, where Type II (ToxoDB #1, #3), and to a lesser extent, Type III (ToxoDB #2) strains predominate in domestic and wild environments (Galal et al., 2019). Looking to Australia, New Zealand's closest neighbour, Type II-representative strains (#1- and #3-like) have been implicated in wildlife toxoplasmosis cases, but also seem to be widespread, having now been identified in animals, humans, and cats in the country (Donahoe et al., 2015; Brennan et al., 2016).

9.3 Further research outcomes

9.3.1 Toxoplasmosis a cause of mortality for cats in New Zealand: an unusual case of toxoplasmic gastritis

Although *T. gondii* infections in cats usually have minimal or no associated clinical signs, toxoplasmosis can be serious and may be considered an important cause of death for these animals (Henriksen et al., 1994; Jokelainen et al., 2012). Feline cases of toxoplasmosis in New Zealand had not previously been described in the literature and warranted investigation to give a complete picture of feline infection in the country. Cases of feline toxoplasmosis drawn from the Pathobiology Group database (School of Veterinary Science, Massey University, New Zealand) were re-examined, and where lesions consistent with toxoplasmosis as well as intra-lesional protozoan-like organisms were seen, further characterisation was carried out using IHC, TEM, and molecular methods. A total of six cats were included in the study. Further characterisation of the cases meant it was possible to give a definitive diagnosis of toxoplasmosis to five of the six cats. For one cat, a diagnosis of suspected toxoplasmosis was given. Clinical signs, gross lesions and histological lesions were generally in agreement with previous studies from overseas. However, one case proved to be particularly interesting, revealing a rare manifestation of toxoplasmosis, namely toxoplasmic gastritis, which was confirmed by IHC and TEM (reviewed by J. Dubey). This manifestation has only previously been reported in one other naturally infected cat overseas (McConnell et al., 2007).

9.3.2 Commercially sourced mussels may be a health risk for consumers

As the study mussels from Chapter 6 were sourced from local supermarkets and therefore destined for human consumption, the objectives were extended to test for *Cryptosporidium* spp. and *Giardia duodenalis*. These are protozoans closely related to *T. gondii*, which are of public health concern in New Zealand. Whilst *Cryptosporidium* spp. was not detected in sampled mussel haemolymph, *G. duodenalis* assemblage B, known to be pathogenic in humans, was discovered in 1 % mussels tested by PCR (n = 90). This thesis therefore represents the first time the presence of potentially pathogenic *T. gondii* and *G.*

duodenalis has been reported in commercially sourced mussels from New Zealand, indicating that these parasites are being transported in sewage, surface or agricultural runoff to rivers and streams, and eventually to coastal waters. Although it is difficult to link protozoal infections to consumption of shellfish, particularly due to long incubation times and lack of symptoms in many people, recreationally sourced mussels have previously been linked to two cases of giardiasis in humans reported in New Zealand (Scholes et al., 2009). Shellfish consumption has also been identified as a significant risk factor for human toxoplasmosis in the United States (OR = 2.22, $p < 0.05$) (Jones et al., 2009) and Taiwan (OR = 3.7, $p = 0.008$) (Chiang et al., 2014). Considering that sporulated *T. gondii* oocysts were confirmed to be present in these supermarket mussels and *T. gondii* DNA was detected in wild-harvested mussels (Chapters 6, 8; Coupe et al., 2018), commercially and recreationally-sourced green-lipped mussels may be a health concern in New Zealand, as the infective stage of these parasites could reach consumers. As such, this thesis recommends that green-lipped mussels and other shellfish in New Zealand are thoroughly cooked to inactivate (oo)cysts before human consumption. Heating *T. gondii*, *G. duodenalis*, and *Cryptosporidium* spp. (oo)cysts at 80 °C for 2 min, at least, is required to render them nonviable (Travaillé et al., 2016; Shapiro et al., 2019a).

9.4 Methods for detecting *T. gondii* along the land-sea transmission chain in New Zealand

Understanding land-sea transmission of *T. gondii* requires both the successful diagnosis of toxoplasmosis or *T. gondii* infection in key hosts and detection of environmental contamination with *T. gondii* oocysts. For these purposes, a variety of tests were employed in thesis, including PCR, serological assays, IHC, and TEM. No one test is perfect, so a particular focus of the thesis was to ensure that the limitations of the tests employed were acknowledged and accounted for through validation and performance evaluation (Chapters 3, 6, 7).

9.4.1 Evaluating the performance of serological assays to determine *T. gondii* seroprevalence in New Zealand cats

Variable performance of different tests in different hosts means that the evaluation of test characteristics – diagnostic sensitivity (Se) and specificity (Sp) – is a necessary prerequisite for interpretation of test results in sero-surveys (Joseph et al., 1995; Greiner & Gardner, 2000; Mainar-Jaime & Barberán, 2007; Pan-ngum et al., 2013; Johnson et al., 2019). An objective of Chapter 3 was therefore to evaluate the performance of the three serological assays for *T. gondii* available in New Zealand, for use in cats. The available tests included a latex agglutination test (LAT) used by veterinary diagnostic labs in the country, a commercial IgG ELISA (IDEXX Chekit Toxotest) for small ruminants, and an in-house western blot (WB). The objective was met by building a novel Bayesian latent class model including three dependent tests, two with binary outcomes (LAT and WB), and one with a continuous outcome (ELISA). The model took into account expert opinion on the diagnostic sensitivity and specificity of each test and considered the tests to be dependent on one another because they all detect anti-*T. gondii* IgG antibodies. Results showed that the ELISA was the best performing test overall with a diagnostic sensitivity and specificity of 84% and 86%, respectively. It is therefore acceptable for use in New Zealand cats but not ideal. Globally, the most sensitive and specific tests available for determining *T. gondii* seroprevalence in cats are reported to be the modified agglutination test (MAT) described by Dubey & Desmonts (1987) and its commercially available counterpart, the Toxo-Screen DA kit (BioMerieux, France) (Tenter et al., 1994). Results of Chapter 3 therefore highlight the need for better reagent and commercial kit availability in New Zealand for future cat testing, preferably permitting the introduction and validation of the MAT or Toxo-Screen DA kit. Nevertheless, by employing Bayesian latent class analysis to take into account the imperfect nature of the available tests, the study was able to provide the first estimate of true *T. gondii* seroprevalence in owned cats in New Zealand.

9.4.2 Validation of PCR methods for use with green-lipped mussels as biosentinels of *T. gondii*

Polymerase chain reaction is the preferred method for detecting *T. gondii* in shellfish, and many other biological samples. There appears to be a general consensus in the literature that PCR assays based on a 529 bp repetitive element in the *T. gondii* genome (rep529) are most sensitive for *T. gondii* detection, no matter the sample type (Edvinsson et al., 2006; Kasper et al., 2009; Yang et al., 2009; Sterkers et al., 2010; Su et al., 2010; Staggs et al., 2015; Wells et al., 2015). There is also a trend towards the use of real-time PCRs, as assays incorporating real-time technologies are more rapid and perhaps more analytically sensitive than alternative methods. They are also considered to be less susceptible to contamination than nested PCRs (Calderaro et al., 2006; Bastien et al., 2008). In the field of *Toxoplasma* research, this has meant that real-time PCRs targeting the rep529 marker are considered the gold standard and these assays have recently been employed in shellfish research in California (Staggs et al., 2015). High analytical sensitivity is particularly important in shellfish research as low concentrations of oocysts are expected in naturally exposed shellfish tissues (Hohweyer et al., 2013). However, currently a variety of PCR-based methods are available to confirm presence of *T. gondii* DNA in marine shellfish, and systematic investigations comparing these molecular methods are scarce. Recent research has also pointed to the importance of using oocyst-spiking experiments when using shellfish as biosentinels of marine contamination with *T. gondii* (Shapiro et al., 2015; Staggs et al., 2015). This is because there are inhibitors specific to shellfish tissues which can interfere with molecular detection of oocyst DNA/RNA, and because the oocyst is unusual amongst the life stages of *T. gondii*, with an extremely hardy outer wall that must be cracked during nucleic acid extraction. The aim of Chapter 7 was therefore to find a suitably rapid and analytically sensitive PCR assay for testing large quantities of wild green-lipped mussel haemolymph, in order to assess the prevalence of *T. gondii* in Hector's dolphin habitat (Chapter 8).

As such, the primary objective of Chapter 7 was to evaluate analytical sensitivity and specificity of a rep529 real-time assay for detection of *T. gondii* DNA in green-lipped mussel (*Perna canaliculus*) haemolymph using

oocyst spiking experiments. Three other assays were also validated for this purpose, including the nPCR *dhps* presently favoured in New Zealand (Roe et al., 2013; Howe et al., 2014; Patel et al., 2019; Chapter 6), the nPCR B1 favoured in California (VanWormer et al., 2014; Shapiro et al., 2015), and a real-time B1 assay. The lowest limit of detection was 5 oocysts using qPCR assays, with the rep529 primers performing best, with good correlation between oocyst concentrations and Cq values, and acceptable efficiency. Considering the wider literature, it is difficult to compare assay performance across studies due to differences in DNA extraction methods, oocyst stock preparation, PCR reagents, amongst others. However, the rep529 assay in this study appeared to perform well in terms of analytical sensitivity compared with other assays previously evaluated in shellfish (Esmerini et al., 2010; Shapiro et al., 2015; Staggs et al., 2015). Assay specificity was evaluated in the study by testing DNA from closely related protozoans, *Hammondia hammondi*, *Neospora caninum*, and *Sarcocystis* spp. Both nPCR assays were specific to *T. gondii*. Both qPCR assays cross-reacted with *Sarcocystis* spp. DNA and the rep529 primers also cross-reacted with *N. caninum* DNA. Findings suggest that until a better assay is developed, the use of the real-time PCR targeting the rep529 described in this study is recommended for testing large numbers of green-lipped mussels for the presence of *T. gondii* DNA, as this was the most sensitive assay, and is relatively time- and cost-effective compared with nested PCRs. However, direct sequencing of positive amplicons is also required, as the assay cross-reacted with *N. caninum* and *Sarcocystis* spp. DNA. Direct sequencing is now generally advised in shellfish studies (Shapiro et al., 2015; Bahia-Oliveira et al., 2017) because popular *T. gondii* PCR targets can also cross-react with other marine organisms, such as DNA from mussels, amoeba, algae, or phytoplankton (Shapiro et al., 2015).

9.5 Main challenges of the thesis

During the course of this research, a number of challenges and unforeseen circumstances arose. This section discusses the main challenge faced by this thesis, namely sample preservation. Sample preservation was, unexpectedly, a recurring issue in this thesis. Challenges arose with long-term storage of samples that appeared to affect downstream molecular analyses (Chapters 2, 5, 8).

9.5.1 Sample preservation issues: FFPE tissues and downstream molecular analyses

In Chapter 2, formalin-fixed tissues from stranded or bycaught New Zealand cetaceans were obtained from the New Zealand Common Dolphin Project (NZCDP) based under the Coastal-Marine Research Group (Massey University, Albany). These tissues were trimmed and embedded in paraffin to form FFPE blocks before being examined for *T. gondii* presence using histological, immunohistochemical, and molecular methods. No evidence of *T. gondii* infection was found in any of dolphins included in the study. Particularly, all samples were negative for *T. gondii* DNA using a nPCR which targets a relatively large fragment of the *dhps* gene (~450 bp in size) (validated in Chapter 7). Whilst FFPE tissues are commonly archived and used for subsequent analyses, it was realised that fixation in formalin can adversely affect the quality of tissue DNA, decrease the average size of extracted DNA fragments, and lead to ineffective target amplification in a PCR (Greer et al., 1991; Douglas & Rogers, 1998; Srinivasan et al., 2002; Ferrer et al., 2007; Dietrich et al., 2013). The duration of fixation is one of the most important factors impacting the extent of DNA degradation (Ferrer et al., 2007; Dietrich et al., 2013) and, in this study, tissues were fixed for an average of 2.16 years before DNA extraction. The possibility that DNA integrity was not optimal for robust PCR amplification of larger targets, like that of the nPCR *dhps*, could therefore not be ignored.

Given this issue, the study was modified in two ways. Firstly, the quality of extracted DNA was assessed by amplifying and sequencing a dolphin species specific housekeeping gene (GAPDH), which was smaller than the *dhps* fragment (~111 bp). This was to serve as an endogenous external amplification control. Bands of expected size were seen for 50 % of lung tissue samples tested, accounting for 50 % of the common dolphins included in the study, and 67 % of striped dolphins. Sequencing was successful for the common dolphin pool, confirmed by NCBI BLAST to have 92 % nucleotide similarity to the GAPDH gene of striped dolphin *Stenella coeruleoalba* (GenBank DQ404538.1; Spinsanti et al., 2006). Secondly, DNA from all dolphin lung samples that showed GAPDH amplification was re-tested for presence of *T. gondii* DNA using a rep529 qPCR targeting a smaller 81 bp fragment of the *T. gondii* genome, which had been validated in

Chapter 7. All lung samples were again found to be negative for *T. gondii*. Overall, results suggested that common, dusky, and striped dolphins in New Zealand have a lower prevalence of *T. gondii* infection and toxoplasmosis than Hector's dolphins. Unfortunately, it was not possible to definitively confirm this or to conclude that *T. gondii* is truly absent in these species, in part due to these specimen preservation issues.

In Chapter 5, a similar problem was faced. This chapter aimed to describe clinical cases of toxoplasmosis in New Zealand domestic cats, using FFPE tissues to carry out IHC, PCR, and TEM for confirmatory purposes. However, as discussed in the chapter, it proved challenging to reach a definitive diagnosis of toxoplasmosis using PCR as a confirmatory tool. It was found that PCR on FFPE tissues can be negative despite an abundance of immunopositive organisms on IHC, and that results were not consistent between replicates, especially for the nPCR *dhps*. Originally, just the nPCR *dhps* was employed in the study. However, PCR amplification of the *T. gondii* B1 gene (nPCR B1) and of the apicomplexan phylum internal transcribed spacer region 1 (ITS1) was also introduced as these alternative markers have greater copy numbers in the *T. gondii* genome, potentially increasing assay sensitivity. The issues faced by Chapter 5 mirror those of Chapter 2 in that age-related DNA fragmentation may have reduced the amplifiable length of a significant proportion of *T. gondii* DNA below the size of the PCR markers used in the study (*dhps* = 450 bp; B1 = 530 bp; ITS-1 = 550 bp). In this instance, however, the problem was more likely to be the age of the FFPE specimens (6 – 20 years), as all tissues had been processed after a maximum fixation time of one week in formalin. Nevertheless, it was ultimately possible to confirm the presence of *T. gondii* DNA in five of the six study cats and reach a definitive diagnosis of toxoplasmosis.

If incorporating molecular work in future studies of this nature, it is therefore ideal to prepare and test FFPE specimens promptly after collection, or to collect an extra set of tissues specifically sampled and stored in a way suitable for nucleic acid preservation. Tissues may be flash frozen, for example, or DNA/RNA

preservation media may be used to ensure sample integrity for downstream molecular analyses, to improve molecular detection and genotyping success. These options, however, were not available in this thesis.

9.5.2 Sample preservation issues: long-term storage of mussel haemolymph and downstream molecular analyses

Sample preservation issues also arose when testing wild-harvested mussel haemolymph for the presence of *T. gondii* DNA after long-term storage (Chapter 8), as mentioned above. From April 2014 to January 2017, over 19 collection dates, 1844 green-lipped mussel haemolymph samples were obtained, and stored at -80°C for 5 – 36 months. The best-performing assay from Chapter 7, the rep529 qPCR, was employed for detection of *T. gondii* DNA in the samples. Unexpectedly, no evidence of the parasite was found in 246 mussels tested. Considering 12.5 % (*n* = 104) of the commercially sourced green-lipped mussels in Chapter 6 were confirmed to be positive for *T. gondii* DNA using the nPCR *dhps* (found in Chapter 7 to be less analytically sensitive than the rep529 qPCR), a high prevalence was anticipated. This presumption was reinforced by results gained from initial wild mussel test batches, collected and tested in April, May, and July 2014. Initial testing found 19.3 % (32/166) of the haemolymph samples were positive for *T. gondii* DNA using the nPCR *dhps*. Surprisingly, 43 mussels which had haemolymph remaining after processing in the initial test batches were found to be negative in the full study, which was conducted approximately 3 years later. Chapter 8 therefore describes steps taken to troubleshoot the discrepancy between initial test batch and full-scale study results, provides results of this process, and explores implications of this work for future studies.

Specifically, human error, the use of an alternative DNA extraction method and PCR assay for *T. gondii* DNA detection in the initial versus full study, were systematically ruled out as the cause of the discrepancy. It was therefore hypothesised that long-term storage of haemolymph negatively affects the ability to detect *T. gondii* DNA in samples. This hypothesis was tested by conducting a storage study, in which new

haemolymph samples were collected from Raglan mussels, which were then either tested fresh (within 72 h), or stored for five months at -80°C before processing, in an effort to mimic the conditions in the full study in which haemolymph samples were stored at -80°C for 5 – 36 months. The same DNA extraction protocol used in the initial test batches was used throughout the storage study, and haemolymph was tested for *T. gondii* DNA using the nPCR *dhps*. Results are discussed above. Chapter 8 concluded with recommendations for continued investigation of *T. gondii* in green-lipped mussels in New Zealand. In particular, DNA should be extracted from green-lipped mussel haemolymph within 72 h of haemolymph collection, until more in-depth studies are carried out to determine optimal haemolymph storage conditions. This is imperative, as until direct testing of seawater becomes feasible, the use of shellfish as bio-sentinels will remain an important tool.

9.6 Summary, recommendations and future studies

9.6.1 Future studies

The studies presented in this thesis have brought to light several avenues for future exploration.

Future shellfish research should consider using probe-based real-time technologies to increase specificity and reduce cross-reactivity with other protozoan parasites in prevalence studies (Chapter 7). Also, adoption of novel molecular tools such as droplet digital PCR (ddPCR) could offer increased sensitivity and specificity despite inhibition problems inherent in shellfish studies (Marino et al., 2019).

Recent research overseas has not only shown that experimentally exposed fish can be vectors of *T. gondii* oocysts (Massie et al., 2010), but has confirmed the presence of *T. gondii* DNA in naturally exposed fish from different levels of the marine food chain (Zhang et al., 2014; Marino et al., 2019). Along these lines, future research in New Zealand could look for evidence of *T. gondii* oocyst presence and accumulation in

Hector's dolphin prey. This would certainly help solidify the land-sea connection and clarify relative exposure risks for the Hectors dolphin and other marine mammals in New Zealand.

An essential line of future research is to confirm the presence of infectious oocysts in shellfish and/or fish sampled from coastal waters in New Zealand. As highlighted, this thesis includes the first study to show presence of sporulated, potentially infectious *T. gondii* oocysts in shellfish, an important step. However, as discussed, the SporoSAG RT-PCR assay used in this study cannot definitively prove infectivity of the *T. gondii* oocysts detected. The new cell-culture assay validated for use in shellfish (Rousseau et al., 2019) seems to be a promising alternative to the gold standard animal bioassay, one which may feasibly be adopted in New Zealand.

It is also essential to further research into *T. gondii* genotyping in New Zealand. A pressing concern for future studies is to determine the source of the variant Type II genotypes linked to fatalities in Hector's dolphins and other wildlife. Genotyping, however, did prove to be difficult throughout this thesis. Future studies should therefore focus on optimising sample collection and preservation so downstream molecular testing and tracking analyses can be successfully be carried out.

Further, efforts should be made to better estimate owned, stray, and feral cat densities in the country. This would directly benefit *T. gondii* research in New Zealand as this data could be used to provide more accurate estimates of oocyst loading on land neighbouring Hector's dolphin habitat. It could also be used to model oocyst transport from land to sea to determine high-risk times and locations for Hector's dolphin exposure to *T. gondii* oocysts (VanWormer et al., 2016; Roberts et al., 2019). This information would also be of wider value in the country; cats, as predators, pose a general threat to many endemic wildlife species in New Zealand.

9.6.2 Recommendations

Ultimately, the most sustainable approach for reducing the risk of *T. gondii* exposure for Hector's dolphins in New Zealand should focus on reducing *T. gondii* contamination at its source, through domestic cat management strategies, as well as mitigating the flow of contaminated runoff to water bodies (Shapiro et al., 2019a). Overall, the studies conducted on the domestic cat in New Zealand showed that *T. gondii* infection is widespread in the country. Owned, stray, and feral cats can all contribute oocysts to the New Zealand environment which may reach nearshore waters (Chapters 3 – 5, 6, 8). For management purposes, companion cats should be neutered to limit breeding, thus reducing the population size and environmental oocyst burden, particularly if future research determines that companion cats are the source of variant Type II oocysts in a domestic *T. gondii* cycle (Chapter 4). Also, although it is a controversial practice, companion cats could be kept indoors to limit the amount of faeces being deposited outside and time spent hunting, considering predation to be main source of infection for cats. Strays should also be neutered, and it is recommended to adopt strays over the purchase of kittens/cats from breeders to further reduce the number of cats in New Zealand. Feral cat control is already being practiced in New Zealand and is likely to remain in practice due to negative impacts of cats on native wildlife through hunting. This should also be considered as an anti-*T. gondii* strategy, especially as feral cats may have a higher prevalence of oocyst shedding than stray and owned cats (Chapter 4). Results of this series of studies also strongly suggest that anti-*T. gondii* cat vaccines should be adopted for all domestic cats when they become available, to limit oocyst shedding (Ramakrishnan et al., 2019).

9.6.3 Summary

To conclude, this thesis investigated land-sea transmission of *T. gondii* in New Zealand in an effort to determine how widespread this parasite is in New Zealand coastal waters and to better understand whether representatives of variant Type II *T. gondii* are particularly pathogenic for the endangered Hector's dolphin, as hypothesised by Roe et al. (2013). Although there were limitations to the research, highlighted above and in the research chapters, several important findings were made which can direct future studies.

Particularly, it was shown that *T. gondii* infection is widespread in New Zealand cats, with trillions of oocysts expected to be shed into the terrestrial environment annually, including in regions adjacent to Hector's dolphin habitat and other areas of conservation importance (Chapters 3, 4). Findings showed that oocysts of *T. gondii* are indeed making their way from land to sea in New Zealand, as evidenced by the detection of sporozoite mRNA in mussel haemolymph samples (Chapter 6). Furthermore, through the use of wild mussels as biosentinels, it was ascertained that *T. gondii* is still present in Māui dolphin habitat and therefore remains a pathogen of concern for the Hector's dolphin species (Chapter 8). Although further confirmatory research is needed, results also suggested that *T. gondii* infection and disease is more prevalent in Hector's dolphins than other cetacean species in New Zealand (Chapter 2). Whether this is due to differences in exposure to *T. gondii* or susceptibility to toxoplasmosis still remains to be resolved, however. Interestingly, the variant Type II *T. gondii* genotype linked to fatal toxoplasmosis in Hector's dolphins was not detected in the thesis and, as such, it is still possible that this genotype is particularly pathogenic for the Hector's dolphin and other endangered wildlife in New Zealand.

9.7 References

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10 Appendices

10.1 Appendix 2i: Immunohistochemistry Protocol for *Toxoplasma gondii* in Dolphin Tissues

Preparation of sections

For all tissues, do the following:

1. Locate paraffin-embedded tissue blocks.
2. Cut and mount 5 μm sections on positively charged slides
3. Allow slides to dry overnight
4. Dewax slides in Leica staining machine (program 7)
5. Place slides in water until ready for antigen retrieval to prevent drying

Positive and negative control tissues

Run with each batch of slides:

1. Positive - brain from a Hector's dolphin with confirmed toxoplasmosis (45670-4, from Roe et al 2013)
2. Positive – lung from a cat with confirmed toxoplasmosis (45286-15)
3. Negative - infected Hector's dolphin brain (45670-4) with omission of primary antibody
4. Negative – infected cat lung (45286-15) with omission of primary antibody

Preparation of reagents

Prepare 10X TBS, 1X TBS, TBS/Triton X-100, 0.3 % H_2O_2, and trypsin antigen retrieval solution, following recipes below

Antigen retrieval – Proteolytic Induced Enzyme Retrieval (PIER)

Using water bath pre-heated to 37 °C

1. Warm slides in 37 °C distilled water for a minimum of 5 min
2. Pour trypsin solution into coplin jar or similar and warm to 37 °C in water bath
3. Add slides to trypsin solution and incubate in water bath for 30 min

Block endogenous enzyme activity/Quenching

1. Lay slides out on a rack over the sink and apply TBS/Triton X-100 ~3 times in 5 min.
2. Place slides in rack and place rack in container filled with 0.3 % H_2O_2, to block endogenous peroxidases. Dip slides in and out gently before leaving for 30 min.

Immunohistochemistry staining

1. Make up 1° antibody diluent, according to recipe.
2. Make up 1° antibody dilution (polyclonal caprine anti-*T.gondii* antibody, VMRD; 1:1000)
3. Wash slides in ~2 mL 1X TBS for 5 min
4. Load slides into the Sequenza
5. Wash with ~2 mL 1X TBS per slide for 5 min
6. Add 150 μl 1° antibody dilution to all slides except negative control
7. Place lid on Sequenza with damp hand towel inside
8. Incubate in fridge <u>overnight</u>
9. Wash with ~2 mL 1X TBS for 5 min
10. Prepare 2 ° antibody diluent, according to recipe
11. Prepare 2 ° antibody dilution (biotinated donkey origin anti-goat IgG, Rockland; 1:200)
12. Incubate slides with 150 μl 2° antibody for 30 min
13. Prepare ABC reagent (Vectastain) and allow to stand 30 min.

14. Wash slides with 1X TBS for 5 min.
15. Incubate slides with 150 μl ABC for 20 min
16. Wash slides with 1X TBS for 5 min.

Chromagen staining

1. Make up DAB (3,3' – diaminobenzidine) in fume hood, sufficient for 175 μl per slide.
2. Remove slides from Sequenza and transfer to slideholder in distilled water.
3. Double-gloved, place slides onto holder over DAB tray under fume hood. Tap off excess water onto paper towel.
4. Cover sections on slides with 175 μl DAB. Focus on tissue and place parafilm over slides.
5. Allow slides to incubate for 4-10 min until they turn a light brown.
6. Rinse off parafilm, transfer slides to slide holder in distilled water
7. Dispose of waste in biohazard rubbish bag in fumehood.
8. Rinse slides under gently running tap water for 5 min.

Counter-staining and coverslipping

Counter-stain slides in Leica staining machine.

1. Select program 6.
2. Place rack in loading drawer and seat properly. Close lid.
3. Select "Load" to start counter-staining process.

Add coverslips using Leica coverslipping machine

1. Load black rack into bottom drawer and close.
2. Ensure adequate coverslips present & rack present for collecting slides once process is completed.
3. Lift needle attachment, press the middle lower button to prime needle.
4. Lift needle attachment and seat it in the back holder. Close lid. Press "Start."

5. Allow coverslips to dry onto slides by leaving slides in horizontal position.

Examine slides

Examine slides for presence of *Toxoplasma gondii*, identified by positively-staining brown antigen-antibody complex.

Recipes

Ensure pH meter has recently been calibrated

10X Tris buffered saline (TBS) stock solution (10X TBS)

Tris Base	14 g
Tris HCl	60 g
Sodium chloride	87.5 g
Distilled water	1000 mL

1X Tris buffered saline (TBS) – 50 mM

Dilute 100 mL 10X TBS with 900 mL distilled water to make 1 L 1X TBS

Ensure pH = 7.6 (± 0.05).

Add 1 M HCl to decrease pH. Add 1 M NaOH to increase pH.

1X TBS containing 0.025 % (v/v) Triton X-100

To make 250 mL (30 slides):

Triton X-100	62.5 uL
1X TBS	250 mL

Trypsin enzymatic antigen retrieval solution

Preheat 200 mL 1X TBS to 37 °C using a water bath

Trypsin 1:250	0.2 g
$CaCl_2$	0.2 g

Mix to dissolve. Allow solution to warm to 37 °C.

Ensure pH = 7.8 (± 0.05).

Add 1 M HCl to decrease pH. Add 1 M NaOH to increase pH.

0.3 % H_2O_2

To make 250 mL (30 slides):

30 % H_2O_2	2.5 mL
1X TBS	247.5 mL

1 ° antibody diluent

Bovine serum albumin	0.1 g
1X TBS	9 mL

Vortex. Top up to 10 mL with 1X TBS.

1 ° antibody dilution

To make 1:1000 dilution:

Anti-*T. gondii* antibody	5 μL	(polyclonal caprine anti-*T.gondii* antibody, VMRD)
Diluent	5000 μL	

2 ° antibody diluent (1X TBS containing 0.01 % (v/v) Triton X-100)

1X TBS	10 mL
Triton X-100	10 μL

2 ° antibody dilution

2 ° antibody	50 μL	(biotinated donkey origin anti-goat IgG, Rockland)

TBS/Triton 0.01 %	10 mL

ABC reagent (Vectastain)

To make 5mL:

Solution A	0.1 mL (2 drops)
Solution B	0.1 mL (2 drops)
1X TBS	5 mL

DAB (3,3'-diaminobenzidine) solution

Handle DAB kit and contents while wearing two pairs of gloves.

Work in fumehood when mixing DAB solution.

To make 5 mL (for 30 slides):

Distilled water	5 mL
Buffer	2 drops
DAB	4 drops
H_2O_2	2 drops

Shake well between adding each ingredient.

Dispose of gloves in rubbish in fume hood.

Apply solution with disposable pipette.

10.2 Appendix 2ii: Dolphin GAPDH PCR

***Dolphin GAPDH* PCR** **(to confirm Dolphin DNA present in FFPE tissues)**

Nucleic acid extraction	**Kit**
DNA	Qiagen DNeasy FFPE kit.

Primers	**Name**	**Sequence (5'-3')**	**Size**	**Position**	**Target**
Forward	GAPDH-F	5'- CAAGGCTGTGGGCAAGGTCATC -3'	~111bp	396 (7th exon)	GAPDH gene GenBank DQ404538
Reverse	GAPDH-R	5'- TTCTCCAGGCGGCAGGTCAG -3'		497 (7th exon)	

PCR kit: Invitrogen Platinum Taq Polymerase

Reagent mix	**First Round Volume (50µL)**
Sterile distilled water	35.3ul
10x PCR buffer	5 ul
MgCl2 (50mM)	1.5 ul
dNTPs (10mM)	1.0 ul
10 µM GAPDH F primer	1.0 ul
10 µM GAPDHR primer	1.0 ul
Taq	0.2 ul
DNA	5.0ul (normalised to 10ng/5ul)
	Description
Positive	"H" samples of Dolphin DNA
Negative	Nuclease free water

Cycling parameters:	Temp (°C)	Time	No. cycles
Hold	94	5 min	1
Denature	95	15 sec	40
Anneal	60	30 sec	
Extension	72	30 sec	
Hold	72	10 min	1
Extension	4	hold	

Electrophoresis

	Description	Size of amplicon(s) (bp)
Agarose gel	1.5%	~111 bp
MW marker	100 bp	

References

Spinsanti G, Panti C, et al. (2006) Selection of reference genes for quantitative RT-PCR studies in striped dolphin (*Stenella coeruleoalba*) skin biopsies. BMC Molecular Biology 7(32)

Buckle K, Roe WD, Howe L, et al. (2017) Brucellosis in endangered Hector's dolphins (*Cephalorhynchus hectori*). Veterinary pathology 54(5), 838-845

10.3 Appendix 2iii: *Toxoplasma gondii* nPCR (*dhps*)

***T. gondii* nPCR (*dhps*):** **(for detection of *T. gondii*)**

Nucleic acid extraction	***Kit***
DNA	*Qiagen DNeasy Blood and Tissue kit.*

Primers	**Name**	**Sequence (5'-3')**	**Size**	**Position**	**Target**
External Forward	FOOD1	GGA ACA TCC GCT GAA GCT CAT GG	490 bp	1169	*dhps* gene GenBank U81497.1 (Pashley et al 1997)
External Reverse	FOOD2	CAG AGA ATC CAG TTG TTT CGA GG		1660	
Internal Forward	FOOD3	CAG TCC AGA CTC GTT CAC CGA TC	420 bp	1201	
Internal Reverse	FOOD4	CCG GAA TAG TGA TAT ACT TGT AG		1616	

PCR Kit: Platinum® Taq DNA Polymerase (Invitrogen)

Reaction Mix	***First Round (25 µL)***
Nuclease-free Water	*17.05 µl*
10X PCR Buffer	*2.5 µl*
$MgCl_2$ (50mM)	*0.75 µl*
dNTP (10mM)	*0.5 µl*
10 µM External Forward Primer (FOOD1)	*0.5 µl*
10 µM External Reverse Primer (FOOD2)	*0.5 µl*
BSA (10 mg/ml)	*0.5 µl*
Taq	*0.2 µl*
DNA	*2.5 µl*

Reaction Mix	***Second Round (50 µL)***
Nuclease-free Water	*34.3µl*
10x PCR Buffer	*5.0 µl*
$MgCl_2$ (50mM)	*1.5 µl*
dNTP (10mM)	*1.0 µl*
10 µM Internal Forward Primer (Pml/S2)	*1.0 µl*
10 µM Internal Reverse Primer (Pml/AS2)	*1.0 µl*
BSA (10 mg/ml)	*0.5 µl*
Taq	*0.2 µl*
DNA	*2.0 µl from First Round*

PCR Controls	***Description***
Positive	Toxovax® DNA (Type I)
Negative	Nuclease free Water

Cycling Parameters First Round	Temp (°C)	Time	No of Cycles
Hold	94	5 min	1
Denature Anneal Extension	94 57 72	30 sec 30 sec 1 min	40
Hold	72 4	10 min ∞	1

Cycling Parameters Second Round	Temp (°C)	Time	No of Cycles
Hold	94	5 min	1
Denature Anneal Extension	94 60 72	30 sec 30 sec 30 sec	40
Hold	72 4	10 min ∞	1

Electrophoresis

	Description	Size of amplicon(s) (bp)
Agarose gel	1.5%	~111 bp
MW marker	100 bp	

References

Aspinall TV, Marlee D, Hyde JE, et al (2002) Prevalence of *Toxoplasma gondii* in commercial meat products as monitored by polymerase chain reaction–food for thought? International Journal for Parasitology 32(9), 1193-1199.

10.4 Appendix 2iv: *Toxoplasma gondii* 529bp Repeat Element qPCR

***Toxoplasma gondii* (529bp Repeat Element (RE)) qPCR**

Nucleic acid extraction	**Kit**
DNA	Qiagen DNEasy Blood and Tissue Kit

Primers	**Name**	**Sequence (5'-3')**	**Amplicon Size**	**Position**	**Target[1]**
Forward	Toxo RE For	CAC AGA AGG GAC AGA AGT CG	81	81	Fragment of 529bp RE
Reverse	Toxo RE rev	CAG TCC TGA TAT CTC TCC TCC AAG		161	

qPCR kit: FastStart Universal SYBR Green Master

Reagent[2]	**Reaction Volume (20uL)**
SYBR Green Master	10.0
PCR grade water	7.6
25uM Toxo RE For	0.2
25uM Toxo RE rev	0.2
Template DNA	2.0

PCR controls	**Description**
Positive	Toxoplasma DNA extracted from Toxovax
Negative	Nuclease-free water

PCR Program Name: Toxo RE qPCR

Cycling Parameters[2]	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	95	10 min	1
Denature	95	15 sec	40
Anneal	54	60 sec	

Electrophoresis	**Description**	**Size of amplicon (bp)**
Agarose gel	1.8 %	81
MW marker	100 bp	

Run: 100V for 40 min

References
1. Kasper et al (2009) Quantitative real-time polymerase chain reaction for the accurate detection of Toxoplasma gondii in amniotic fluid. *Diagnostic Microbiology and Infectious Disease* 63, pp. 10-15.
2. FastStart Universal SYBR Green Master (ROX) Instructions (2008) Version 04

10.5 Appendix 2v Formalin fixation time estimates for dolphin tissues

Formalin fixation time estimates (days) for dolphin tissues provided by the New Zealand Common Dolphin Project (NZCDP) based under the Coastal-Marine Research Group (Massey University, Albany, New Zealand). Data was only available for common dolphins (*Delphinus* spp.). Mean = 787 days

Dolphin Species	Tissue Collection Date	Trimming Date	Formalin fixation time (days)
Delphinus delphis New Zealand common dolphin			
1	18-Aug-2008	22-Jul-2013	1799
2	25-Aug-2008	24-Jul-2013	1794
3	20-Jul-2009	18-Jul-2013	1459
4	31-Jul-2009	18-Jul-2013	1448
5	31-Jul-2009	24-Jul-2013	1454
6	20-Jan-2010	23-Jul-2013	1280
7	15-May-2010	22-Jul-2013	1164
8	22-Sep-2010	18-Jul-2013	1030
9	14-Feb-2011	5-Sep-2013	934
10	9-May-2011	19-Jul-2013	802
11	13-May-2011	19-Jul-2013	798
12	17-Jun-2011	18-Jul-2013	762
13	19-Aug-2011	24-Jul-2013	705
14	18-Nov-2011	22-Jul-2013	612
15	8-Aug-2012	24-Jul-2013	350
16	7-Aug-2012	5-Sep-2013	394
17	28-Aug-2012	24-Jul-2013	330

18	29-Aug-2012	5-Sep-2013	372
19	7-Dec-2012	5-Sep-2013	272
20	21-Jan-2014	13-Jun-2016	874
21	20-Jan-2014	10-Jun-2016	872
22	30-Jan-2014	13-Jun-2016	865
23	21-Jan-2014	10-Jun-2016	871
24	22-Jan-2014	13-Jun-2016	873
25	22-Jan-2014	13-Jun-2016	873
26	10-Jul-2014	9-Jun-2016	700
27	10-Oct-2014	10-Jun-2016	609
28	17-Oct-2014	13-Jun-2016	605
29	17-Oct-2014	9-Jun-2016	601
30	5-Jun-2015	13-Jun-2016	374
31	5-Jun-2015	13-Jun-2016	374
32	1-Jul-2015	13-Jun-2016	348
33	27-Jul-2015	9-Jun-2016	318
34	27-Jul-2015	10-Jun-2016	319
35	14-Aug-2015	10-Jun-2016	301

10.6 Appendix 3i: JAGS code for Bayesian latent class model

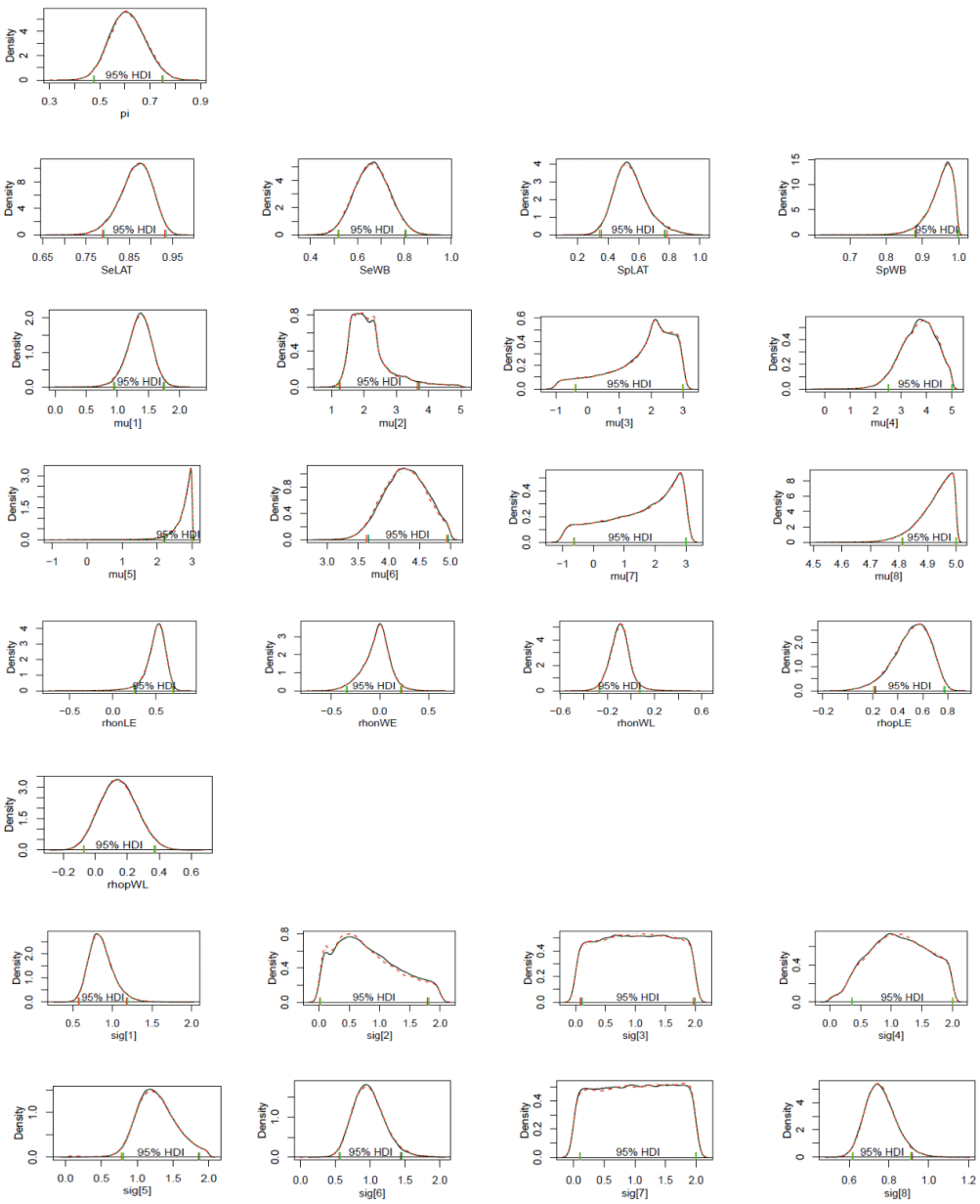
```
model{

for (i in 1:n)    {

z[i]~dbern(pi)                                    #infection status of
cat i

pWB[i]<-z[i]*SeWB+(1-z[i])*(1-SpWB)            #observed prevalance
P(WB+)

WB[i]~dbern(pWB[i])


w1[i]<-WB[i]+1                                      # 1 if WB=0, 2 if
WB=1

pLAT[i]<-z[i]*SeLATc[w1[i]]+(1-z[i])*(1-SpLATc[w1[i]])

                                    #Se/SpLATc are conditional on WB
                                    result

LAT[i]~dbern(pLAT[i])


zwl[i]<-1+z[i]+2*WB[i]+4*LAT[i]              # 1 to 8 depending on z,
WB, LAT

lnE[i]~dnorm(mu[zwl[i]],tau[zwl[i]])           #assume lnE normal
conditional on z, WB, LAT

}


#Derived parameters:

SeLAT<-(1-SeWB)*SeLATc[1]+SeWB*SeLATc[2]

SpLAT<-SpWB*SpLATc[1]+(1-SpWB)*SpLATc[2]

rhopWL<-SeWB*(SeLATc[2]-SeLAT)/sqrt(SeWB*SeLAT*(1-SeWB)*(1-SeLAT))

rhonWL<-(1-SpWB)*(SpLAT-SpLATc[2])/sqrt(SpWB*SpLAT*(1-SpWB)*(1-SpLAT))


pp[1] <- (1-SeWB)*(1-SeLATc[1] )   #WB=0,LAT=0
```

```
pp[2] <- SeWB*(1-SeLATc[2])        #10

pp[3] <- (1-SeWB)*SeLATc[1]        #01

pp[4] <- SeWB*SeLATc[2]            #11


pn[1] <- SpWB*SpLATc[1]             #00

pn[2] <- (1-SpWB)*SpLATc[2]         #10

pn[3] <- SpWB*(1-SpLATc[1] )        #01

pn[4] <- (1-SpWB)*(1-SpLATc[2])     #11


EplnE<-mu[2]*pp[1]+mu[4]*pp[2]+mu[6]*pp[3]+mu[8]*pp[4]

EpWlnE<-mu[4]*pp[2]+mu[8]*pp[4]

EpLlnE<-mu[6]*pp[3]+mu[8]*pp[4]

VplnE<-
mu[2]*mu[2]*pp[1]+mu[4]*mu[4]*pp[2]+mu[6]*mu[6]*pp[3]+mu[8]*mu[8]*pp[4]+


sig[2]*sig[2]*pp[1]+sig[4]*sig[4]*pp[2]+sig[6]*sig[6]*pp[3]+sig[8]*sig[8
]*pp[4] - EplnE*EplnE

rhopWE<-(EpWlnE-EplnE*SeWB)/sqrt(SeWB*(1-SeWB)*VplnE)

rhopLE<-(EpLlnE-EplnE*SeLAT)/sqrt(SeLAT*(1-SeLAT)*VplnE)


EnlnE<-mu[1]*pn[1]+mu[3]*pn[2]+mu[5]*pn[3]+mu[7]*pn[4]

EnWlnE<-mu[3]*pn[2]+mu[7]*pn[4]

EnLlnE<-mu[5]*pn[3]+mu[7]*pn[4]

VnlnE<-
mu[1]*mu[1]*pn[1]+mu[3]*mu[3]*pn[2]+mu[5]*mu[5]*pn[3]+mu[7]*mu[7]*pn[4]+


sig[1]*sig[1]*pn[1]+sig[3]*sig[3]*pn[2]+sig[5]*sig[5]*pn[3]+sig[7]*sig[7
]*pn[4] - EnlnE*EnlnE

rhonWE<-(EnWlnE-EnlnE*(1-SpWB))/sqrt(SpWB*(1-SpWB)*VnlnE)

rhonLE<-(EnLlnE-EnlnE*(1-SpLAT))/sqrt(SpLAT*(1-SpLAT)*VnlnE)
```

```
#ROC and AUC:

for (k in 1:60)   {

c[k]<- -1+k*0.1

se[k]<-1-(pp[1]*phi((c[k]-mu[2])/sig[2])+pp[2]*phi((c[k]-
mu[4])/sig[4])+pp[3]*phi((c[k]-mu[6])/sig[6])+

pp[4]*phi((c[k]-mu[8])/sig[8]))

fp[k]<-1-(pn[1]*phi((c[k]-mu[1])/sig[1])+pn[2]*phi((c[k]-
mu[3])/sig[3])+pn[3]*phi((c[k]-mu[5])/sig[5])+

pn[4]*phi((c[k]-mu[7])/sig[7]))

}

#se and fp at cutoff of 30

c30<-log(30+1)

se30<-1-(pp[1]*phi((c30-mu[2])/sig[2])+pp[2]*phi((c30-
mu[4])/sig[4])+pp[3]*phi((c30-mu[6])/sig[6])+

pp[4]*phi((c30-mu[8])/sig[8]))

fp30<-1-(pn[1]*phi((c30-mu[1])/sig[1])+pn[2]*phi((c30-
mu[3])/sig[3])+pn[3]*phi((c30-mu[5])/sig[5])+

pn[4]*phi((c30-mu[7])/sig[7]))

#se and fp at optimal cutoff (o)

co<-log(30.9+1)

seo<-1-(pp[1]*phi((co-mu[2])/sig[2])+pp[2]*phi((co-
mu[4])/sig[4])+pp[3]*phi((co-mu[6])/sig[6])+

pp[4]*phi((co-mu[8])/sig[8]))

fpo<-1-(pn[1]*phi((co-mu[1])/sig[1])+pn[2]*phi((co-
mu[3])/sig[3])+pn[3]*phi((co-mu[5])/sig[5])+

pn[4]*phi((co-mu[7])/sig[7]))

#AUC<-step(lnEp-lnEn)


#priors:
```

```
pi~dbeta(9.6816,14.0224)                    # mode = 0.4, 95 sure > 0.25

SeWB~dbeta(12.3804, 5.6483)                 # mode = 0.71, 95 sure > 0.5

SpWB~dbeta(6.252, 1.9268)                   # mode = 0.85, 95 sure > 0.5

SeLATc[1]~dbeta(11.5581,5.1059)             # mode = 0.72, 95 sure > 0.5
SeLATc[2]~dbeta(11.5581,5.1059)             # mode = 0.72, 95 sure > 0.5
SpLATc[1]~dbeta(6.0514,1.8223)              # mode = 0.86, 95 sure > 0.5
SpLATc[2]~dbeta(6.0514,1.8223)              # mode = 0.86, 95 sure > 0.5

for (j in 1:4)    {
mu[2*j-1]~dunif(-1,3)
mu[2*j]~dunif(mu[2*j-1],5)
}
for (j in 1:8)          {
tau[j]<-1/sig[j]/sig[j]
sig[j]~dunif(0,2)
}

}
```

10.7 Appendix 3ii: Posterior plots from Bayesian latent class analysis



10.8 Appendix 3iii: Sensitivity Analysis

```
#priors

pi~dbeta(7.4783,9.5875)                # mode = 0.43, 95 sure > 0.25


SeWB~dbeta(10.8353, 4.6377)            # mode = 0.73, 95 sure > 0.5

SpWB~dbeta(6.9618, 2.3087)             # mode = 0.82, 95 sure > 0.5

SeLATc[1]~dbeta(10.1963, 4.2311)       # mode = 0.74, 95 sure > 0.5
SeLATc[2]~dbeta(10.1963, 4.2311)       # mode = 0.74, 95 sure > 0.5
SpLATc[1]~dbeta(6.7051, 2.1685)        # mode = 0.83, 95 sure > 0.5
SpLATc[2]~dbeta(6.7051, 2.1685)        # mode = 0.83, 95 sure > 0.5

for (j in 1:4) {
mu[2*j-1]~dunif(-1,3)
mu[2*j]~dunif(mu[2*j-1],5)
}
for (j in 1:8)         {
tau[j]<-1/sig[j]/sig[j]
sig[j]~dunif(0,2)
}

Iterations = 6001:11000
Thinning interval = 1
Number of chains = 3
Sample size per chain = 5000

1. Empirical mean and standard deviation for each variable,
   plus standard error of the mean:

            Mean      SD  Naive SE Time-series SE
pi      0.673327 0.07938 0.0006481      0.0033723

SeWB    0.617505 0.07526 0.0006145      0.0028614
SpWB    0.930649 0.04361 0.0003561      0.0008936

SeLAT   0.874658 0.03803 0.0003105      0.0008106
SpLAT   0.625233 0.13175 0.0010757      0.0058414

mu[1]   1.331925 0.21596 0.0017633      0.0039072
mu[2]   2.145090 0.61668 0.0050352      0.0155332
mu[3]   1.797505 0.95552 0.0078018      0.0155524
mu[4]   3.715155 0.68901 0.0056257      0.0099562
mu[5]   2.518410 0.55313 0.0045163      0.0238061
mu[6]   4.099766 0.33334 0.0027217      0.0118892
mu[7]   1.576329 1.14177 0.0093225      0.0165164
mu[8]   4.927057 0.05889 0.0004808      0.0008785
```

```
rhonLE  0.416813 0.19339 0.0015790      0.0075108
rhonWE  0.004595 0.16829 0.0013741      0.0032541
rhonWL -0.058898 0.11198 0.0009143      0.0034482
rhopLE  0.484780 0.14815 0.0012096      0.0037893
rhopWE  0.431712 0.11371 0.0009285      0.0032830
rhopWL  0.134660 0.11520 0.0009406      0.0025284
sig[1]  0.864928 0.16714 0.0013647      0.0027672
sig[2]  0.794146 0.49303 0.0040256      0.0135229
sig[3]  1.024009 0.56499 0.0046132      0.0082644
sig[4]  1.110016 0.47961 0.0039160      0.0078179
sig[5]  1.254368 0.34760 0.0028382      0.0087288
sig[6]  1.048471 0.21267 0.0017364      0.0056260
sig[7]  1.013842 0.57078 0.0046604      0.0062385
sig[8]  0.758614 0.07709 0.0006295      0.0009257

2. Quantiles for each variable:

           2.5%      25%      50%       75%  97.5%
pi      0.51761  0.61758  0.67379  0.731643 0.8185

SeWB    0.48267  0.56377  0.61314  0.667019 0.7744
SpWB    0.82406  0.90839  0.93962  0.962236 0.9884

SeLAT   0.79098  0.85108  0.87830  0.901744 0.9392
SpLAT   0.39945  0.52616  0.61266  0.717030 0.8953

mu[1]   0.85790  1.20475  1.34596  1.476253 1.7179
mu[2]   1.38923  1.73769  2.01620  2.342234 3.9927
mu[3]  -0.60208  1.31372  2.07453  2.510424 2.9533
mu[4]   2.22839  3.25898  3.76964  4.226476 4.8694
mu[5]   0.91307  2.37086  2.69753  2.873351 2.9886
mu[6]   3.49896  3.85742  4.08111  4.334178 4.7767
mu[7]  -0.79615  0.72530  1.86347  2.581117 2.9666
mu[8]   4.78017  4.89514  4.94081  4.973540 4.9976
rhonLE -0.12099  0.34770  0.46262  0.542526 0.6602
rhonWE -0.35744 -0.09285  0.01343  0.105425 0.3431
rhonWL -0.24634 -0.12985 -0.06901 -0.005189 0.2123
rhopLE  0.15441  0.39170  0.49909  0.592687 0.7337
rhopWE  0.17574  0.36415  0.44466  0.512839 0.6179
rhopWL -0.08379  0.05470  0.13416  0.213492 0.3639
sig[1]  0.59495  0.75112  0.84636  0.958352 1.2421
sig[2]  0.05934  0.41000  0.70983  1.121925 1.8701
sig[3]  0.05410  0.55467  1.04283  1.505523 1.9505
sig[4]  0.23610  0.73674  1.10213  1.499263 1.9385
sig[5]  0.48757  1.03932  1.24544  1.485322 1.9140
sig[6]  0.66337  0.90084  1.03776  1.183099 1.4952
sig[7]  0.06005  0.52229  1.01385  1.503157 1.9517
sig[8]  0.62110  0.70494  0.75416  0.806654 0.9255
```

```
Iterations = 11001:16000
Thinning interval = 1
Number of chains = 3
Sample size per chain = 5000

1. Empirical mean and standard deviation for each variable,
   plus standard error of the mean:

          Mean       SD  Naive SE Time-series SE
fp[1]  0.98885 0.011984 9.785e-05      2.427e-04
fp[2]  0.98663 0.013623 1.112e-04      2.774e-04
fp[3]  0.98397 0.015465 1.263e-04      3.225e-04
fp[4]  0.98079 0.017498 1.429e-04      3.671e-04
fp[5]  0.97698 0.019712 1.610e-04      4.043e-04
fp[6]  0.97243 0.022144 1.808e-04      4.589e-04
fp[7]  0.96700 0.024811 2.026e-04      5.206e-04
fp[8]  0.96054 0.027724 2.264e-04      5.858e-04
fp[9]  0.95289 0.030881 2.521e-04      6.628e-04
fp[10] 0.94389 0.034268 2.798e-04      7.743e-04
fp[11] 0.93335 0.037869 3.092e-04      8.995e-04
fp[12] 0.92112 0.041657 3.401e-04      1.011e-03
fp[13] 0.90703 0.045603 3.723e-04      1.144e-03
fp[14] 0.89093 0.049666 4.055e-04      1.280e-03
fp[15] 0.87268 0.053814 4.394e-04      1.427e-03
fp[16] 0.85224 0.058005 4.736e-04      1.586e-03
fp[17] 0.82958 0.062200 5.079e-04      1.756e-03
fp[18] 0.80473 0.066345 5.417e-04      1.936e-03
fp[19] 0.77779 0.070408 5.749e-04      2.123e-03
fp[20] 0.74892 0.074355 6.071e-04      2.245e-03
fp[21] 0.71835 0.078152 6.381e-04      2.437e-03
fp[22] 0.68634 0.081751 6.675e-04      2.630e-03
fp[23] 0.65321 0.085105 6.949e-04      2.818e-03
fp[24] 0.61931 0.088170 7.199e-04      2.988e-03
fp[25] 0.58500 0.090899 7.422e-04      3.105e-03
fp[26] 0.55064 0.093219 7.611e-04      3.368e-03
fp[27] 0.51652 0.095086 7.764e-04      3.514e-03
fp[28] 0.48297 0.096415 7.872e-04      3.634e-03
fp[29] 0.45019 0.097177 7.934e-04      3.610e-03
fp[30] 0.41833 0.097316 7.946e-04      3.688e-03
fp[31] 0.38732 0.096898 7.912e-04      3.725e-03
fp[32] 0.35746 0.095961 7.835e-04      3.764e-03
fp[33] 0.32931 0.094603 7.724e-04      3.792e-03
fp[34] 0.30266 0.093041 7.597e-04      3.815e-03
fp[35] 0.27779 0.090948 7.426e-04      3.790e-03
fp[36] 0.25467 0.088175 7.199e-04      3.706e-03
fp[37] 0.23311 0.084919 6.934e-04      3.582e-03
```

```
fp[38] 0.21300 0.081325 6.640e-04      3.429e-03
fp[39] 0.19425 0.077518 6.329e-04      3.276e-03
fp[40] 0.17676 0.073551 6.005e-04      3.093e-03
fp[41] 0.16060 0.069544 5.678e-04      2.901e-03
fp[42] 0.14566 0.065527 5.350e-04      2.703e-03
fp[43] 0.13185 0.061540 5.025e-04      2.501e-03
fp[44] 0.11910 0.057622 4.705e-04      2.299e-03
fp[45] 0.10733 0.053803 4.393e-04      2.101e-03
fp[46] 0.09650 0.050107 4.091e-04      1.910e-03
fp[47] 0.08655 0.046551 3.801e-04      1.727e-03
fp[48] 0.07743 0.043148 3.523e-04      1.564e-03
fp[49] 0.06909 0.039906 3.258e-04      1.379e-03
fp[50] 0.06149 0.036829 3.007e-04      1.267e-03
fp[51] 0.05459 0.033918 2.769e-04      1.097e-03
fp[52] 0.04833 0.031173 2.545e-04      9.680e-04
fp[53] 0.04269 0.028592 2.334e-04      8.580e-04
fp[54] 0.03760 0.026169 2.137e-04      7.588e-04
fp[55] 0.03304 0.023903 1.952e-04      6.699e-04
fp[56] 0.02897 0.021787 1.779e-04      5.905e-04
fp[57] 0.02533 0.019816 1.618e-04      5.166e-04
fp[58] 0.02211 0.017984 1.468e-04      4.518e-04
fp[59] 0.01925 0.016287 1.330e-04      3.972e-04
fp[60] 0.01672 0.014718 1.202e-04      3.489e-04
fp30   0.11499 0.056311 4.598e-04      2.231e-03
se[1]  0.99936 0.001298 1.060e-05      1.933e-05
se[2]  0.99925 0.001460 1.192e-05      2.200e-05
se[3]  0.99912 0.001641 1.340e-05      2.502e-05
se[4]  0.99898 0.001842 1.504e-05      2.845e-05
se[5]  0.99881 0.002067 1.688e-05      3.248e-05
se[6]  0.99861 0.002318 1.893e-05      3.695e-05
se[7]  0.99838 0.002599 2.122e-05      4.204e-05
se[8]  0.99811 0.002911 2.377e-05      4.785e-05
se[9]  0.99779 0.003261 2.663e-05      5.510e-05
se[10] 0.99743 0.003652 2.982e-05      6.286e-05
se[11] 0.99700 0.004090 3.339e-05      7.180e-05
se[12] 0.99650 0.004579 3.739e-05      8.210e-05
se[13] 0.99591 0.005128 4.187e-05      9.477e-05
se[14] 0.99524 0.005742 4.688e-05      1.086e-04
se[15] 0.99444 0.006430 5.250e-05      1.246e-04
se[16] 0.99352 0.007199 5.878e-05      1.430e-04
se[17] 0.99244 0.008058 6.579e-05      1.643e-04
se[18] 0.99119 0.009015 7.360e-05      1.888e-04
se[19] 0.98973 0.010079 8.229e-05      2.115e-04
se[20] 0.98804 0.011258 9.192e-05      2.410e-04
se[21] 0.98606 0.012562 1.026e-04      2.805e-04
se[22] 0.98377 0.013999 1.143e-04      3.214e-04
se[23] 0.98110 0.015576 1.272e-04      3.674e-04
se[24] 0.97800 0.017300 1.413e-04      4.187e-04
se[25] 0.97435 0.019158 1.564e-04      4.739e-04
se[26] 0.96993 0.021315 1.740e-04      5.401e-04
```

```
se[27] 0.96506 0.023800 1.943e-04      6.256e-04
se[28] 0.95992 0.026192 2.139e-04      7.075e-04
se[29] 0.95441 0.028537 2.330e-04      7.875e-04
se[30] 0.94850 0.030859 2.520e-04      8.666e-04
se[31] 0.94217 0.033107 2.703e-04      9.412e-04
se[32] 0.93533 0.035193 2.873e-04      1.004e-03
se[33] 0.92785 0.037087 3.028e-04      1.055e-03
se[34] 0.91989 0.039072 3.190e-04      1.116e-03
se[35] 0.91235 0.041242 3.367e-04      1.200e-03
se[36] 0.90487 0.043358 3.540e-04      1.288e-03
se[37] 0.89710 0.045444 3.711e-04      1.377e-03
se[38] 0.88887 0.047524 3.880e-04      1.468e-03
se[39] 0.88001 0.049611 4.051e-04      1.563e-03
se[40] 0.87040 0.051703 4.222e-04      1.660e-03
se[41] 0.85988 0.053792 4.392e-04      1.758e-03
se[42] 0.84829 0.055836 4.559e-04      1.947e-03
se[43] 0.83544 0.057785 4.718e-04      2.046e-03
se[44] 0.82124 0.059682 4.873e-04      2.142e-03
se[45] 0.80550 0.061462 5.018e-04      2.232e-03
se[46] 0.78800 0.063054 5.148e-04      2.336e-03
se[47] 0.76846 0.064317 5.251e-04      2.398e-03
se[48] 0.74695 0.065360 5.337e-04      2.392e-03
se[49] 0.72320 0.066033 5.392e-04      2.419e-03
se[50] 0.69712 0.066280 5.412e-04      2.421e-03
se[51] 0.66864 0.066043 5.392e-04      2.429e-03
se[52] 0.63776 0.065274 5.330e-04      2.385e-03
se[53] 0.60457 0.063932 5.220e-04      2.315e-03
se[54] 0.56922 0.062011 5.063e-04      2.220e-03
se[55] 0.53194 0.059519 4.860e-04      2.100e-03
se[56] 0.49320 0.056597 4.621e-04      1.964e-03
se[57] 0.45348 0.053296 4.352e-04      1.815e-03
se[58] 0.41322 0.049734 4.061e-04      1.658e-03
se[59] 0.37299 0.046047 3.760e-04      1.507e-03
se[60] 0.33334 0.042378 3.460e-04      1.358e-03
se30   0.81608 0.060304 4.924e-04      2.173e-03

2. Quantiles for each variable:

             2.5%      25%     50%     75%   97.5%
fp[1]  0.9557631 0.985549 0.99250 0.99650 0.99944
fp[2]  0.9490508 0.982539 0.99068 0.99546 0.99917
fp[3]  0.9414684 0.979077 0.98843 0.99413 0.99880
fp[4]  0.9330949 0.974878 0.98570 0.99244 0.99828
fp[5]  0.9239112 0.969911 0.98235 0.99029 0.99752
fp[6]  0.9132006 0.964030 0.97829 0.98754 0.99649
fp[7]  0.9017508 0.957216 0.97331 0.98409 0.99509
fp[8]  0.8885281 0.949044 0.96732 0.97981 0.99329
fp[9]  0.8729179 0.939416 0.96011 0.97448 0.99089
fp[10] 0.8559460 0.928265 0.95147 0.96799 0.98758
fp[11] 0.8364529 0.915423 0.94131 0.96014 0.98338
```

```
fp[12] 0.8155587 0.900641 0.92921 0.95068 0.97795
fp[13] 0.7931024 0.883863 0.91532 0.93943 0.97097
fp[14] 0.7688339 0.864995 0.89938 0.92632 0.96275
fp[15] 0.7415164 0.843905 0.88130 0.91131 0.95206
fp[16] 0.7126737 0.820739 0.86117 0.89381 0.93954
fp[17] 0.6819567 0.794874 0.83875 0.87401 0.92457
fp[18] 0.6483839 0.767392 0.81412 0.85215 0.90749
fp[19] 0.6128193 0.737939 0.78738 0.82767 0.88763
fp[20] 0.5763757 0.706580 0.75894 0.80135 0.86628
fp[21] 0.5390979 0.673095 0.72892 0.77340 0.84357
fp[22] 0.5012946 0.638497 0.69713 0.74439 0.81833
fp[23] 0.4620010 0.602898 0.66428 0.71356 0.79179
fp[24] 0.4214989 0.566165 0.63068 0.68205 0.76343
fp[25] 0.3836251 0.529598 0.59626 0.65028 0.73481
fp[26] 0.3463265 0.492741 0.56175 0.61787 0.70478
fp[27] 0.3094635 0.457143 0.52733 0.58542 0.67556
fp[28] 0.2738626 0.421864 0.49340 0.55311 0.64526
fp[29] 0.2409843 0.388386 0.46047 0.52079 0.61475
fp[30] 0.2094545 0.355607 0.42813 0.48910 0.58489
fp[31] 0.1800531 0.324202 0.39682 0.45788 0.55324
fp[32] 0.1548443 0.293947 0.36695 0.42777 0.52235
fp[33] 0.1315778 0.265491 0.33804 0.39884 0.49231
fp[34] 0.1101746 0.238483 0.31108 0.37130 0.46313
fp[35] 0.0909124 0.214258 0.28650 0.34544 0.43454
fp[36] 0.0750495 0.192127 0.26320 0.32085 0.40603
fp[37] 0.0612324 0.172229 0.24124 0.29661 0.37832
fp[38] 0.0497443 0.153913 0.22061 0.27429 0.35261
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fp[53] 0.0012808 0.019171 0.03964 0.06209 0.10529
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fp[56] 0.0005409 0.011046 0.02522 0.04260 0.08006
fp[57] 0.0004069 0.009098 0.02147 0.03731 0.07256
fp[58] 0.0002874 0.007473 0.01820 0.03265 0.06588
fp[59] 0.0002076 0.006102 0.01539 0.02846 0.05963
fp[60] 0.0001498 0.004948 0.01295 0.02461 0.05407
fp30   0.0120769 0.071001 0.11798 0.15764 0.21676
```

```
se[1]  0.9955352 0.999356 0.99988 0.99999 1.00000
se[2]  0.9949545 0.999221 0.99985 0.99999 1.00000
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se[4]  0.9936401 0.998857 0.99974 0.99997 1.00000
se[5]  0.9928741 0.998609 0.99967 0.99996 1.00000
se[6]  0.9919403 0.998318 0.99958 0.99995 1.00000
se[7]  0.9909616 0.997970 0.99947 0.99993 1.00000
se[8]  0.9898182 0.997566 0.99932 0.99990 1.00000
se[9]  0.9885389 0.997072 0.99915 0.99986 1.00000
se[10] 0.9871077 0.996502 0.99892 0.99980 1.00000
se[11] 0.9853938 0.995826 0.99865 0.99973 1.00000
se[12] 0.9837727 0.995048 0.99831 0.99964 1.00000
se[13] 0.9816835 0.994166 0.99790 0.99951 1.00000
se[14] 0.9793584 0.993120 0.99740 0.99935 1.00000
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se[20] 0.9592799 0.982433 0.99123 0.99675 0.99990
se[21] 0.9544671 0.979612 0.98938 0.99588 0.99984
se[22] 0.9488291 0.976402 0.98721 0.99476 0.99974
se[23] 0.9427613 0.972617 0.98469 0.99335 0.99959
se[24] 0.9360363 0.968419 0.98171 0.99153 0.99937
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se[30] 0.8799342 0.928830 0.95307 0.97214 0.99458
se[31] 0.8688936 0.920995 0.94659 0.96751 0.99274
se[32] 0.8582610 0.912767 0.93978 0.96214 0.99013
se[33] 0.8468683 0.904108 0.93206 0.95586 0.98711
se[34] 0.8359170 0.894709 0.92371 0.94910 0.98383
se[35] 0.8244787 0.885211 0.91618 0.94296 0.98094
se[36] 0.8135034 0.875985 0.90842 0.93707 0.97784
se[37] 0.8015643 0.866589 0.90039 0.93097 0.97462
se[38] 0.7901371 0.856874 0.89212 0.92449 0.97075
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se[40] 0.7640424 0.835251 0.87333 0.90917 0.96079
se[41] 0.7502426 0.823072 0.86268 0.90030 0.95492
se[42] 0.7359646 0.809626 0.85083 0.89016 0.94814
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se[45] 0.6856562 0.761857 0.80710 0.85111 0.91842
se[46] 0.6660135 0.743204 0.78906 0.83452 0.90496
se[47] 0.6447449 0.722705 0.76874 0.81571 0.88875
se[48] 0.6226797 0.700052 0.74649 0.79454 0.87050
se[49] 0.5985387 0.675635 0.72213 0.77079 0.84982
se[50] 0.5729574 0.649472 0.69543 0.74408 0.82579
```

```
se[51] 0.5459286 0.621088 0.66659 0.71507 0.79812
se[52] 0.5171757 0.590838 0.63566 0.68332 0.76740
se[53] 0.4875572 0.558519 0.60245 0.64918 0.73185
se[54] 0.4559880 0.524980 0.56684 0.61232 0.69272
se[55] 0.4233982 0.489460 0.52935 0.57338 0.65045
se[56] 0.3902538 0.452860 0.49073 0.53221 0.60666
se[57] 0.3565176 0.415571 0.45092 0.49034 0.56057
se[58] 0.3224371 0.377881 0.41092 0.44716 0.51359
se[59] 0.2887601 0.340386 0.37079 0.40444 0.46660
se[60] 0.2557928 0.303599 0.33124 0.36223 0.41989
se30   0.6975741 0.773269 0.81793 0.86099 0.92615
```

```
> print(dic.pD)
Mean deviance:  611.1
penalty 65.95
Penalized deviance: 677.1
```

10.9 Appendix 5: *Toxoplasma gondii* nPCR B1 gene

***T. gondii* nPCR (B1)** **(for detection and genotyping of *T. gondii*)**

Nucleic acid extraction	***Kit***
DNA	*Qiagen DNeasy Blood and Tissue kit.*

Primers	**Name**	**Sequence (5'-3')**	**Size**	**Position**	**Target**
External Forward	Pml/S1	5'-TGT TCT GTC CTA TCG CAA CG -3'	530 bp	128-147	B1 gene GenBank AF179871 (Burg 1989)
External Reverse	Pml/AS1	5'-TCT TCC CAG ACG TGG ATT TC -3'		152-171	
Internal Forward	Pml/S2	5'-ACG GAT GCA GTT CCT TTC TG -3'		707-688	
Internal Reverse	Pml/AS2	5'-CTC GAC AAT ACG CTG CTT GA -3'		682-663	

PCR Kit: Platinum® Taq DNA Polymerase (Invitrogen)

Reaction Mix	***First Round (25 µL)***
Nuclease-free Water	*15.55µl*
10X PCR Buffer	*2.5 µl*
$MgCl_2$ (50mM)	*0.75 µl*
dNTP (10mM)	*0.5 µl*
10 µM External Forward Primer (Pml/S1)	*1.0 µl*
10 µM External Reverse Primer (Pml/AS1)	*1.0 µl*
BSA (1 mg/ml)	*1.0 µl*
Taq	*0.2 µl*
DNA	*2.5 µl*

Reaction Mix	***Second Round (50 µL)***
Nuclease-free Water	*34.3µl*
10x PCR Buffer	*5.0 µl*
$MgCl_2$ (50mM)	*1.5 µl*
dNTP (10mM)	*1.0 µl*
10 µM Internal Forward Primer (Pml/S2)	*2.0 µl*
10 µM Internal Reverse Primer (Pml/AS2)	*2.0 µl*
BSA (1 mg/ml)	*2.0 µl*
Taq	*0.2 µl*
DNA	*2.0 µl from First Round*

PCR Controls	**Description**
Positive	Toxovax® DNA (Type I)
Negative	Nuclease free Water

PCR Program Name: B1 nPCR R1/R2

Cycling Parameters First Round	**Temp (°C)**	**Time**	**No of Cycles**
Hold	94	2 min	1
Denature Anneal Extension	94 60 72	30 sec 30 sec 45 sec	35
Hold	72 4	10 min ∞	1

Cycling Parameters Second Round	**Temp (°C)**	**Time**	**No of Cycles**
Hold	94	2 min	1
Denature Anneal Extension	94 60 72	30 sec 30 sec 45 sec	35
Hold	72 4	10 min ∞	1

10.10 Appendix 6i: Coupe et al. (2018)

First report of *Toxoplasma gondii* sporulated oocysts and *Giardia duodenalis* in commercial green-lipped mussels (*Perna canaliculus*) in New Zealand

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Abstract

Pollution of marine ecosystems with the protozoan parasites *Toxoplasma gondii*, *Cryptosporidium* spp., and *Giardia duodenalis* can be studied using bivalve shellfish as biosentinels. Although evidence suggests these parasites are present in New Zealand coastal waters, the extent of protozoal pollution has not been investigated. This study used optimised molecular methods to detect the presence of *Cryptosporidium* spp., *Giardia duodenalis*, and *Toxoplasma gondii* in commercially-sourced green-lipped mussel (*Perna canaliculus*), an endemic species found throughout coastal New Zealand. A nested polymerase chain reaction was validated for detection of *T. gondii* DNA and applied to 104 commercially-sourced mussels. Thirteen mussels were positive for *T. gondii* DNA with an estimated true prevalence of 16.4 % using Bayesian statistics and the presence of *T. gondii* in mussels was significantly associated with collection during the summer compared with the winter ($P = 0.003$). Consumption of contaminated shellfish may also pose a health risk for humans and marine wildlife. As only sporulated *T. gondii* oocysts can be infectious, a reverse-transcriptase polymerase chain reaction was used to confirm presence of a sporozoite-specific marker (SporoSAG), detected in four mussels. *Giardia duodenalis* assemblage B, known to be pathogenic in humans, was also discovered in 1 % mussels, tested by polymerase chain reaction ($n = 90$). *Cryptosporidium* spp. was not detected in the sampled mussel haemolymph. Results suggest that New Zealand may have high levels of coastal contamination with *T. gondii*, particularly in summer months, and that naturally-exposed mussels can ingest and retain sporulated oocysts, further establishing shellfish consumption as a health concern.

Keywords: biosentinels; *Toxoplasma gondii*; *Giardia duodenalis*; SporoSAG; *Perna canaliculus*

Introduction

Toxoplasma gondii, *Cryptosporidium* spp., and *Giardia* spp. are water-borne protozoan parasites of significant medical and veterinary importance. Found worldwide, (oo)cysts of these parasites are released into the environment, often in vast quantities. *Cryptosporidium* spp. and *Giardia duodenalis* (oo)cysts are shed in faeces of a variety of infected animals, including humans (Fayer et al. 1998; Thompson 2004) but *T. gondii* oocysts are only excreted by cats, primarily after first infection (Dabritz and Conrad 2010). Freshwater sources contaminated with *Cryptosporidium hominis*, *C. parvum* or *Giardia duodenalis* (synonyms *G. intestinalis* and *G. lamblia*) (oo)cysts are well-associated with human outbreaks of diarrhoea, which can be recurrent and severe (Miller et al. 2005; Lucy et al. 2008; Hohweyer et al. 2013; Willis et al. 2013). Drinking water contaminated with *T. gondii* oocysts has been linked to outbreaks of acute toxoplasmosis in humans (Bowie et al. 1997; Jones and Dubey 2010). Human infection with *T. gondii* can also be serious, particularly for pregnant women, congenitally-infected children, and immunosuppressed people, resulting in encephalitis, chorioretinitis, and abortion (Tenter et al. 2000; Sukthana 2006). An estimated 30 % of the entire human population is chronically infected with *T. gondii* and latent infection has been linked to a number of psychological disorders (Tenter et al. 2000; Webster et al. 2013; Ene et al. 2016; but see Sugden et al. 2016).

Water-sampling studies show that highly resistant (oo)cysts of *Cryptosporidium* and *Giardia* spp. can pollute estuarine and marine ecosystems, likely transported in freshwater surface runoff (Johnson et al. 1995; Fayer 2004; Betancourt et al. 2014). Whilst direct detection of *T. gondii* in seawater is more challenging (Karanis et al. 2013; Wells et al. 2015), this parasite is believed to contaminate the coastal environment in a comparable manner (Miller et al. 2002; Conrad et al. 2005; Dubey and Jones 2008; VanWormer et al. 2013, 2016). A number of studies have shown that all three protozoans are present in estuarine and marine shellfish (Fayer et al. 1998; Miller et al. 2005a; Willis et al. 2013; Shapiro et al. 2014, Staggs et al. 2015). Bivalve shellfish such as mussels, oysters, clams, and cockles are filter feeders that concentrate suspended particles, including pathogens, from water. Due to this ability to remove and concentrate waterborne contaminants, bivalves are now recognised worldwide as potential bio-indicators of aquatic pollution (Graczyk et al. 1999; Miller et al. 2005b; Shapiro et al. 2014) and may

further represent a significant health risk to consumers (Downey and Graczyk 2007; Gilbert et al. 2007; Robertson 2007; Jones and Dubey 2010; Smith and Nichols 2010; Chiang et al. 2014).

In New Zealand there is very limited information on *T. gondii*, *Cryptosporidium* and *Giardia* spp. in the marine environment and presence of these parasites has not yet been investigated in shellfish (Gilbert et al. 2007). Previous studies have shown that *Cryptosporidium* and *Giardia* spp. are prevalent in freshwater sources (Brown et al. 1992; Ionas et al. 1998; Till et al. 2008) and consumption of recreationally-sourced shellfish has been linked to two cases of giardiasis in humans reported in the country (Scholes et al. 2009). Toxoplasmosis has recently been identified as a cause of mortality for the endangered Hector's dolphin (*Cephalorhyncus hectori*), found only in New Zealand coastal waters (Roe et al. 2013), but little data have been gathered on human toxoplasmosis in New Zealand. Shellfish consumption has, however, been identified as a significant risk factor for human toxoplasmosis in the United States (OR = 2.22, $p < 0.05$) (Jones et al. 2009) and Taiwan (OR = 3.7, $p = 0.008$) (Chiang et al. 2014). The green-lipped mussel (*Perna canaliculus*) is an endemic shellfish species that occurs naturally throughout coastal New Zealand and these mussels are commonly harvested recreationally for human consumption. In addition, *P. canaliculus* is farmed commercially in New Zealand, and it is estimated that over 63, 000 metric tons of green-lipped mussels are consumed annually by New Zealanders alone (King and Lake 2013). In order to investigate possible links between human consumption of green-lipped mussels and protozoal infections, a molecular assay was validated for the detection of *T. gondii* DNA in mussel haemolymph. The validated test and three additional molecular assays, each detecting a different gene target, were then used to investigate the prevalence of potentially zoonotic protozoans *Toxoplasma gondii*, *Cryptosporidium* spp. and *Giardia duodenalis,* in commercially- sourced green-lipped mussels.

Materials and Methods

Mussel Sampling and Processing. A total of 104 green-lipped mussels (*Perna canaliculus*) that had been commercially grown for human consumption in ocean farms were collected fresh from eight

different commercial outlets in New Zealand at seven time points between September 2013 and November 2015 (see Table 1). It was not possible to obtain details on seller collection dates or sources. Mussels were kept cool and transported to Massey University, Palmerston North, New Zealand, where they were processed within 24 h of collection. Outer shell surfaces were washed by hand with tapwater before a notch was filed to allow aseptic extraction of haemolymph from the posterior adductor muscle using a sterile 22-gauge needle (Shapiro et al. 2014). Aspirated haemolymph was stored in sterile Eppendorf tubes at -20°C for DNA/RNA extraction and PCR analysis. Before processing, haemolymph was thawed and 100 µl haemolymph per mussel was centrifuged at 20,000 x *g* for 10 mins, the supernatant removed, and the haemocyte cell pellet suspended in 100 µl PBS (1X, pH 7.4).

DNA Extraction. DNA was extracted from individual mussel samples using DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany), following procedure detailed in Shapiro et al. (2014). Extraction controls using PBS (1X, pH 7.4) were included. DNA was stored at -20°C until used for molecular analysis. Not all mussels had sufficient haemolymph remaining for DNA extraction and testing for presence of *Cryptosporidium* spp. and *Giardia duodenalis*.

Detection of* Cryptosporidium *spp. and* Giardia duodenalis *DNA by Nested PCR. Nested and semi-nested PCR assays were used to amplify fragments of the 60kDa glycoprotein (GP60) gene of *C. hominis* and *C. parvum* (800 – 850 bp) and the glutamate dehydrogenase (*gdh*) gene found in all assemblages of *G. duodenalis* (432 bp) (Table 2). PCRs were performed as previously described (Garcia-R et al. 2017). All nPCR runs included positive and negative controls. Reactions were visualised on ethidium bromide stained 1 % agarose gels. Positive amplicons were purified by ethanol precipitation and submitted to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequences were analysed using Geneious software R10 (Biomatters, Auckland, New Zealand) and compared with reference sequences for *Cryptosporidium* species and *G. duodenalis* assemblages using a BLAST search (GenBank database, NCBI).

Detection of* T. gondii *DNA by Nested PCR. A spiking experiment was conducted in order to determine the sensitivity of the *T. gondii* nested PCR (nPCR) used in this study (Shapiro et al. 2014). Haemolymph, from green-lipped mussels previously determined to be negative for *T. gondii* DNA in duplicate runs of the nested PCR used in this study, was pooled and divided into 100 µl aliquots. *T. gondii* oocysts (Type II, M4 strain, originally obtained from Lee Innes of the Moredun Research Institute, Edinburgh, Scotland) were quantified and a ten-fold serial dilution prepared using sterile PBS (1X, pH 7.4). Dilutions were added to haemolymph aliquots at concentrations of 1000, 100, 50, 10, 5, and 1 oocyst(s) per 100 µl haemolymph. DNA was extracted for each oocyst concentration and the *T. gondii* nPCR assay run in triplicate per extraction.

A nPCR assay targeting a 450 bp fragment of the *T. gondii dhps* gene using FOOD1/2 and FOOD3/4 primers (Table 2) was employed to detect *T. gondii* DNA, as described in detail by Aspinall et al. (2002a), with the addition of 0.5 µl bovine serum albumin (BSA, 10 mg/ml) to each PCR reaction mix. A positive control from a known *T. gondii* isolate (incomplete strain S48, Toxovax®, MSD Animal Health, Wellington, New Zealand; (Hartley and Bridge 1975)), and a no template control (NTC) using ultrapure water were included in all nPCR runs. Assay specificity was determined by using DNA extracted from closely related protozoans, *Hammondia hammondi*, *Neospora caninum* and *Sarcocystis* spp. To confirm successful amplification, 10 µl of the final PCR product was run on a 1.5% agarose gel containing SYBR Safe (Thermo Fisher Scientific, Boston, MA, USA) and visualised with UV light using an E-Gel Imager (Life Technologies, Carlsbad, CA, USA). Positive amplicons were purified using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA) and submitted to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems). Sequences were analysed using Geneious software 8.1.5 (Biomatters) and compared with reference sequences for *T. gondii* using a BLAST search (GenBank database, NCBI).

Genotyping* T. gondii *– nPCR-RFLP. The *T. gondii dhps* fragment amplified in the nPCR above is not suitable for genotyping as it is too highly conserved between strains (Aspinall et al. 2002a, b; Meneceur et al. 2008). Therefore nPCR-RFLP of the single-copy loci 3'SAG2, GRA6, and SAG1, and of the multi-copy B1 locus was attempted using previously described PCR protocols (Su et al. 2010; Shapiro et al. 2014).

RNA extraction. RNA was extracted from mussel haemolymph which tested positive for *T. gondii* DNA and which had sufficient haemolymph remaining. Extraction was carried out using RNeasy FFPE kits (Qiagen) following manufacturer's instructions with the following modifications. One hundred microliters of haemolymph was pelleted and resuspended in PBS, as described above, before addition of PKD Buffer (150 µl) and immersion in liquid nitrogen for 4 min/boiling water for 4 min. RNA was eluted in 25 µl RNase-free water. Total RNA yield and purity was reviewed using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific).

Detection of* T. gondii *SporoSAG mRNA by RT-PCR. A reverse-transcriptase PCR (RT-PCR) assay targeting a 71 bp fragment of the SporoSAG gene was utilised to amplify sporozoite-specific *T. gondii* mRNA using the primers SporoSAG F 5'-CGG ACA AAT GTG GCG TAC AC-3' and SporoSAG R 5'-GTG ATC TTG CGC CGA ACA C-3' (Travaillé et al. 2016) (Table 2). RT-PCR was performed using the SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen). Each 25 µl reaction mixture was prepared on ice and contained 2X Reaction Mix (12.5 ul), SuperScript® III RT/Platinum® *Taq* Mix (1 µl), 10 uM SporoSAG F (0.5 µl), 10 uM SporoSAG R (0.5 µl), RNA (5 µl; 30 – 50 ng RNA per reaction), and RNase-free water. Controls included a water blank, total RNA extracted from 1000 *T. gondii* tachyzoites and from 1000 sporulated, heat-inactivated (80^{o}C for 20 min), *T. gondii* oocysts. Cycling parameters were as follows: 55°C for 30 min (1 cycle); 94°C for 2min (1 cycle); 94°C for 15 sec, 60°C for 30 sec, 68°C for 45 sec (45 cycles); 68°C for 5 min (1 cycle). To confirm successful amplification, 10 µl of the final PCR product was run on a 2.5 % agarose gel stained with SYBR Safe (Thermo Fisher Scientific), before visualisation, as described above. The size of PCR amplicons was estimated by comparison with the O'RangeRuler 20 bp – 300 bp DNA Ladder (Thermo Fisher Scientific). SporoSAG mRNA was not detected in total RNA extracted from 1000 tachyzoites. However, a positive amplicon was observed from 1000 sporulated oocysts and purified using a PureLink PCR purification kit (Invitrogen). Sequencing confirmed the amplicon to have 100 % nucleotide similarity to *T. gondii* sporozoite-specific SAG protein mRNA (GenBank AY492338.1, Radke et al. 2004). For mussel samples, presence of a band

of expected size was taken as qualitative evidence of successful amplification of *T. gondii* SporoSAG mRNA, as amplicon products were too weak for successful sequencing.

***Statistical Analysis*.** The diagnostic (epidemiological) sensitivity of the nPCR *dhps* assay and true prevalence (as opposed to apparent prevalence given by PCR results) of *T. gondii* were estimated using a Bayesian and Markov Chain Monte Carlo (MCMC) modelling approach (Branscum et al. 2005). Diagnostic specificity was assumed to be 100 % as all positive results were confirmed by direct sequencing. Prior information about the diagnostic sensitivity was modelled using a beta-distribution elicited from a technical expert [L. Howe], assuming a most likely value of 80% and 95% confidence that it is at least 60%. A beta-distribution was also specified for true prevalence informed by overseas prevalence studies, assuming a most likely value of 1.6% and 95% confidence that it is less than 50% (Miller et al. 2008; Esmerini et al. 2010; Putignani et al. 2011; Aksoy et al. 2014; Marangi et al. 2015; Marquis et al. 2015; Shapiro et al. 2014) (Table 3). Analyses were carried out in Betabuster (University of California, Davis, USA) and OpenBUGS version 3.2.3 rev 1012 software to give a posterior median point estimate and 95 % credible interval for both parameters. Elsewhere, 95 % confidence intervals are given using the Wilson method for binomial proportion data. A generalised linear model with a logit link (family = binomial) was used to determine the risk factors for a mussel being *T. gondii* positive. Univariate analyses were performed for source origin (n = 8), year, and season (spring (September, October, November), summer (December, January, February), fall (March, April, May), winter (June, July, August)). Interactions were explored with model simplification using X^2 test to compare nested models and odds ratios estimated from the exponent of the coefficients. Low samples sizes and collinear variables caused singularities for models with interaction terms, but the multivariate analyses supported the univariate results. Significance was indicated when $P \leq 0.05$ for all statistical analyses. These analyses were performed in R version 3.4.2 (R Core Team 2017) using base or plotrix (Lemon 2006), MASS (Venables and Ripley 2002) and binom (Sundar Dorai-Raj 2014) packages.

Results

Cryptosporidium *spp. and* G. duodenalis *in Commercial Mussels.* *G. duodenalis* DNA was found in 1/90 (1.1 %; 95 % Confidence Interval: 0.06 – 6.9) mussel haemolymph samples, with 99 % nucleotide

similarity to *G. duodenalis* Assemblage B at the *gdh* locus (GenBank L40508, Monis et al. 2009). *Cryptosporidium* spp. DNA was not detected in any mussels sampled in the study (Table 1).

Analytical sensitivity and specificity of the nested dhps PCR assay for T. gondii detection. Specificity of the primers for detection of T. gondii was evaluated by testing DNA of closely related protozoans, Hammondia hammondi, Neospora caninum and Sarcocystis spp. Gel electrophoresis showed that the nPCR assay specifically amplified the dhps gene of T. gondii and not the three closely related protozoans. The sensitivity of the assay was determined to be 66.7 % for 50 T. gondii oocysts in mussel haemolymph (2/3 PCR replicates) and 100 % for 100 and 1000 oocysts in mussel haemolymph (3/3 PCR replicates).

T. gondii *in Commercial Mussels.* A total of 13 mussels (12.5 %; 95 % Confidence Interval: 7.09 – 20.8) tested positive for *T. gondii* DNA using the validated nPCR (Table 1). All 13 positive amplicons were confirmed by NCBI BLAST to have 99 – 100 % nucleotide similarity to the *T. gondii dhps* gene (GenBank U81497, Pashley et al. 1997). True prevalence, given by the posterior median point estimate, was calculated to be 16.4 % (95 % Credible Interval: 9.1 – 27.5). Diagnostic sensitivity was calculated to be 77.3 % (95 % Credible Interval: 56.1 – 91.9). A significant difference in *T. gondii* prevalence was observed between mussels sampled in summer, compared with winter, with the odds of detection 15 times higher (95 % Confidence Interval 3 – 113, $P = 0.003$) given the winter baseline (Fig. 1). Amplification of SAG1, GRA6, 3'SAG2, and B1 loci was not successful for any of the 13 mussels with confirmed *T. gondii* DNA. Further molecular characterisation using restriction fragment length polymorphism (RFLP) was therefore not possible. Seven positive samples had sufficient haemolymph remaining for RNA extraction and testing by reverse-transcriptase PCR (RT-PCR). Sporozoite-specific SporoSAG mRNA was detected in four of the seven mussels tested.

Table 1

Date Sampled	No. Positive/No. Tested (%, 95 % Confidence Interval)		
	Toxoplasma gondii	***Cryptosporidium* spp.**	***Giardia duodenalis***
Sep 2013	4/12 (33.3, 14 - 61)	nd	nd
Mar 2014	0/9 (0.0, 0 - 30)	0/9 (0.0, 0 - 30)	0/9 (0.0, 0 - 30)
Jun 2014	2/11 (18.2, 5 - 48)	0/10 (0.0, 0 - 28)	0/10 (0.0, 0 - 28)
Jul 2014	0/11 (0.0, 0 -26)	0/11 (0.0, 0 - 26)	0/11 (0.0, 0 - 26)
Jan 2015	6/14 (42.9, 21 - 67)	0/14 (0.0, 0 - 22)	0/14 (0.0, 0 - 22)
Jul 2015	0/19 (0.0, 0 - 17)	0/19 (0.0, 0 - 17)	0/19 (0.0, 0 - 17)
Nov 2015	1/28 (3.6, 1 - 18)	0/27 (0.0, 0 - 12)	1/27 (3.7, 1 - 18)
Total	**13/104 (12.5, 8 – 20)**	**0/90 (0.0, 0 - 4)**	**1/90 (1.1, 0 - 6)**

Table 2

Protozoan	Gene Target	Primer Name	Primer Sequence	Annealing Temp (°C)	Size (bp)	Reference
T. gondii	nPCR *dhps*	FOOD1	GGA ACA TCC GCT GAA GCT CAT GG	57		Aspinall et al. 2002a
		FOOD2	CAG AGA ATC CAG TTG TTT CGA GG			
		FOOD3	CAG TCC AGA CTC GTT CAC CGA TC	57	450	
		FOOD4	CCG GAA TAG TGA TAT ACT TGT AG			
	RT-PCR SporoSAG	SporoSAG F	CGG ACA AAT GTG GCG TAC AC	60	71	Travaillé et al. 2016
		SporoSAG R	GTG ATC TTG CGC CGA ACA C			
Cryptosporidium spp.	nPCR GP60	AL3531	ATA GTC TCC GCT GTA TTC	57		Peng et al. 2001, Alves et al. 2003
		AL3535	GGA AGG AAC GAT GTA TCT			
		AL3532	TCC GCT GTA TTC TCA GCC	60	800 - 850	
		AL3534	GCA GAG GAA CCA GCA TC			
G. duodenalis	snPCR *gdh*	GDHeF	TCA ACG TYA AYC GYG GYT TCC GT	56		Read et al. 2004
		GDHiF	CAG TAC AAC TCY GCT CTC GG		432	
		GDHiR	GTT RTC CTT GCA CAT CTC C			

Table 3

Parameter	Prior estimate	5th/95th percentile
True prevalence	0.016	< 0.5
Sensitivity	0.8	> 0.6
Specificity	1.0	-

Fig. 1

Discussion

This is the first report of zoonotic protozoan parasites, *G. duodenalis* and *T. gondii*, in commercially-sourced green-lipped mussels grown in New Zealand, providing evidence that green-lipped mussels harbour potentially pathogenic protozoan (oo)cysts.

These results suggest that green-lipped mussels may be a good sentinel species for assessing marine contamination with protozoan pathogens in New Zealand. Previous studies have shown that the detection of (oo)cysts in seawater is challenging (Toze et al. 1999; Verant et al. 2014) and an alternative approach is to sample bivalve shellfish as biological sentinels, which has been used to monitor other waterborne pollutants such as heavy metals and biotoxins (Arkush et al. 2003; Lindsay et al. 2004; Miller et al. 2005b; Shapiro et al. 2014; Kerambrun et al. 2016). Green-lipped mussels can filter up to 9 L of seawater per hour (James et al. 2001) and are able to select food particles on basis of size ranging from 5 – 20 µm (Shumway et al. 1985; Safi and Gibbs 2003). Thus, (oo)cysts of *G. duodenalis* and *T. gondii*, as well as *Cryptosporidium* spp., are of optimum size for *P. canaliculus*, measuring 10 – 12 µm, 7 – 14 µm ,and 4 – 6 µm, respectively (Dubey et al. 1970; Gómez-Couso et al. 2003a; Willis et al. 2013).

Giardia duodenalis DNA was detected in 1 % of mussels tested (1/90, 95 % Confidence Interval: 0 - 6). Molecular characterisation showed that detected DNA belonged to *G. duodenalis* assemblage B, which is known to be pathogenic in humans (Feng and Xiao 2011). Assemblage B has previously been found in *Ostrea edulis* oysters from Spain (Gómez-Couso et al. 2004) and *Mytilus californianus* mussels from California (Adell et al. 2014). *Giardia* as a genus is made up of six species, but giardiasis in humans and most other mammals is caused by *G. duodenalis*, which is a complex of at least eight genetic assemblages (A – H) (Monis et al. 2003; Feng and Xiao 2011; Ryan and Cacciò 2013). So far, only

assemblages A and B are known to be pathogenic in humans (Homan et al. 1998; Read et al. 2004) but have been isolated from a variety of other hosts, including livestock, cats, dogs, and wild mammals (Li et al. 2012). In New Zealand assemblage B is the dominant cause of human giardiasis and has been detected in domestic, introduced wild and zoo animals (Garcia-R et al. 2017). Although it is difficult to link protozoal infections to consumption of shellfish, particularly due to long incubation times and lack of symptoms in many people (Robertson 2007), an outbreak of giardiasis was identified in the United States associated with oyster consumption (Iwamoto et al. 2010) and consumption of recreationally-sourced mussels has been linked to two cases of giardiasis in humans reported in New Zealand between 1997 and August 2004 (Scholes et al. 2009).

The prevalence of *Giardia*-contaminated shellfish reported in this study is relatively low compared with that seen in shellfish in some overseas locations, for example, 10 % of commercial mussels from Italy (*n* = 60, Giangaspero et al. 2014) and 41 % from Spain (*n* = 184, Gómez-Couso et al. 2005) tested positive for this protozoan. This could be an issue of sample size and sampling frequency but could also be because most studies in other regions test multiple tissues using immunofluorescence antibody assays alone or combined with PCR and may employ immunomagnetic separation to concentrate the parasites in tissue homogenates before testing (Miller et al. 2005; Willis et al. 2013; Adell et al. 2014). It is therefore possible that prevalence was underestimated in this study because only haemolymph was tested and a concentration step was not included.

New Zealand has a higher incidence of human giardiasis than most developed countries, with a notification rate of 32.9 cases per 100,000 population for 2015 (ESR 2016). The disease is mainly thought to be transmitted from person to person in New Zealand (Snel et al. 2009), although an increasing number of reports show that dairy cattle can act as reservoirs (Hunt et al. 2000, Learmonth et al. 2003; Winkworth et al. 2008; Abeywardena et al. 2012). Cysts are immediately infectious, can rapidly accumulate in the environment, and survive for weeks to months in soil, freshwater and seawater (Brown et al. 1999; Olson et al. 1999; Miller et al. 2005a, b; Robertson 2007). Thus, the discovery of *Giardia* cysts in freshwater sources in New Zealand (Ionas et al. 1998; Till et al. 2008) and now in green-lipped mussels, suggests that this parasite is being transported in sewage, surface or agricultural runoff to rivers and streams, and eventually to New Zealand coastal waters.

True prevalence of *T. gondii* DNA in mussels tested (*n* = 104) was estimated to be 16.4 %, which was a higher proportion than expected. Mussels were sampled throughout the year over a two-year period. Previous studies that also tested marine shellfish throughout the year have reported a *T. gondii* prevalence of 0.07 % (Miller et al. 2008), 1.4 % (Shapiro et al. 2014), 1.7 % (Marquis et al. 2015) in the USA, 3.3 % (Esmerini et al. 2010) and 8.2 % (Ribeiro et al. 2015) in South America, 3.2 % (Putignani et al. 2011) and 9.4 % in Europe (Aksoy et al. 2014). Seasonal differences have been observed in California, USA, where rainy season sampling appears to be associated with high *T. gondii* prevalence in mussels (Shapiro et al. 2014), even reaching 46. 3 % in a recent study (*n* = 41, Staggs et al. 2015). In locations such as California, USA, most months are dry, and rainfall is needed to flush oocysts into nearshore waters, resulting in increased levels of marine contamination during the wet season (winter, spring) (Shapiro et al. 2014; VanWormer et al. 2016). Here, in contrast, the odds of detecting T. gondii were found to be 15 times higher in mussels sampled in summer, as compared with winter (*P* = 0.003). On average, New Zealand rainfall is generally higher in winter than in summer (NIWA), so this was an unexpected finding that may be explained by region-specific weather patterns seen in the country (NIWA). It is also possible that higher levels of marine pollution and mussel contamination in summer months were due to drought events followed by intense rainfall. Extended dry periods may lead to greater oocyst accumulation on land that can mobilise into overland runoff with subsequent periods of heavy rain (Shapiro et al. 2012; Lal et al. 2013). Seasonal differences in T. gondii prevalence may further be linked to seasonal variations in terrestrial contamination, for example, the timing of the feline breeding season (VanWormer et al. 2013), but this remains to be determined.

Variation in *T. gondii* prevalence between studies may reflect real differences in environmental contamination or seasonal effects. However, it may also be impacted by disparities in test sensitivity, as no standardised protocol for testing shellfish has yet been agreed upon in the literature. Here, a nested PCR targeting the *dhps* gene of *T. gondii* (Aspinall et al. 2002a) was chosen as it has previously been used to investigate *T. gondii* in livestock (Patel et al. 2017 in press) and native wildlife (Roe et al. 2013, 2017; Howe et al. 2014) in New Zealand. Analytical sensitivity of the method for use with mussel haemolymph was established by conducting an oocyst spiking experiment. Only three prior investigations have also used oocyst spiking experiments rather than genomic DNA or DNA from

tachyzoites to determine sensitivity (Esmerini et al. 2010; Shapiro et al. 2014; Staggs et al. 2015). Authors report detection limits of 100 oocysts in mussel tissue homogenate (Esmerini et al. 2010), 5 oocysts in mussel haemolymph (Shapiro et al. 2014), and even a single oocyst in mussel haemolymph (Staggs et al. 2015). In comparison, the nPCR *dhps* in this study was able to detect 50 oocysts in haemolymph. Sensitivity differences may be due to a number of factors, particularly PCR target copy number, shellfish species and tissues tested, and nucleic acid extraction methods. For example, assays targeting the *rep529* gene as used by Staggs et al. (2015) are purportedly the most sensitive (Su et al. 2010) because there are approximately 200 – 300 copies of this marker in the *T. gondii* genome (Homan et al. 2000, Costa and Bretagne 2012). It is not yet known whether the *dhps* gene is single- or multi-copy. It is therefore likely that prevalence was underestimated in this study, notably because contaminated shellfish are thought to only contain low oocyst numbers (Esmerini et al. 2010; Shapiro et al. 2014; Staggs et al. 2015).

We thought it would be interesting to attempt to estimate true prevalence through use of Bayesian statistics to take into account that true *T. gondii* contamination status of each mussel was not known, and that the PCR assay was not 100 % sensitive (Branscum et al. 2005). Incorporating prior information and observed data to describe the uncertainty surrounding prevalence and sensitivity, the posterior distribution produced an estimate of true prevalence of 16.4 % (95 % Credible Interval: 9.1 – 27.5). Bayesian methods generated an estimate of the mean of the posterior distribution for PCR sensitivity of 77 % (95 % Credible Interval: 56.1 – 91.9). Although the Bayesian true prevalence does not appear to be significantly different from apparent prevalence (12.5 % is found within the credible interval for true prevalence), around a quarter of *T. gondii*-contaminated mussels could yield negative test outcomes using this assay.

The relatively high prevalence of *T. gondii* DNA in green-lipped mussels seen in this investigation may be indicative of a large environmental burden of *T. gondii* oocysts in New Zealand. Cats are the only known source of *T. gondii* oocysts, as the definitive host of the parasite. Sexual replication of *T. gondii* occurs in the cat intestine and during active infection a single cat can shed up to 810 million oocysts into the environment in its faeces, over a period no longer than 3 weeks (Dabritz et al. 2006). Studies in the USA suggest that the environmental load of oocysts depends, in part, on the proportion of owned

cats that defecate outdoors (Dabritz and Conrad 2010). For example, Dabritz and Conrad (2010) predicted that oocyst contamination of soil may be 10 times greater in Central and South America due to 80 % of outdoor cats compared to 8 – 17 % in North America. In New Zealand there is a large owned cat population in excess of 1.1 million (New Zealand Companion Animal Council 2016) and it is estimated that at least 90 % are allowed outdoors (Farnworth et al. 2010; Hall et al. 2016). At present, there is no available data on *T. gondii* seroprevalence or oocyst shedding rates in New Zealand cats. However, outdoor cats are likely to have a greater exposure to *T. gondii* through hunting and consumption of birds, rodents and other intermediate hosts, potentially increasing the incidence of cat infections and thus oocyst shedding. New Zealand also has specific climatic and topographical features which may facilitate oocyst transport from land to sea (VanWormer et al. 2016). Particularly, New Zealand is a long narrow country with a high coastline to land mass ratio. Accordingly, a high proportion of river systems discharge at coastlines rather than at inland water bodies. The climate is generally temperate, with relatively high rainfall (NIWA), thought to drive overland runoff events and transport pathogens to coastal waters (Fayer et al. 2004; Shapiro et al. 2014; VanWormer et al. 2016). Mussels in this study originated from ocean farms so prevalence should reflect marine contamination with *T. gondii*. However, high prevalence could result from cross-transmission of oocysts between mussels in holding tanks, as seen experimentally with *Cryptosporidium* spp. oocysts in shellfish (Gómez-Couso et al. 2003b). The green-lipped mussels used in this study were purchased from local supermarkets where large numbers of mussels are held together. Transmission of contamination may therefore be possible, especially if water is recirculated.

T. gondii infections are regularly reported in terrestrial mammals in New Zealand (e.g. West 2002; Patel et al. 2017 in press) and have also been reported in marine mammals (Roe et al. 2013, 2016) and birds (Howe et al. 2014). Comparison between genotypes from the terrestrial and marine environments would help clarify routes and mechanisms of land-sea transmission (Fayer et al. 2004; VanWormer et al. 2014). Unfortunately, genotyping was not possible in this study, likely due to low concentrations of parasite DNA in mussel samples and PCR methods lacking the necessary sensitivity to reliably detect low copy numbers of typing loci in shellfish tissues (Esmerini et al. 2010; Shapiro et al. 2014; Staggs et al. 2015). While the nested *dhps* PCR method used in the current study confirms the presence of *T.*

gondii DNA, it does not distinguish between unsporulated and sporulated oocysts. This is of importance with respect to disease transmission, since only sporulated *T. gondii* oocysts can be infectious (Dubey et al. 1998). To investigate this, we used a RT-PCR targeting the sporozoite-specific SporoSAG gene (Radke et al. 2004; Villegas et al. 2010; Travaillé et al. 2016) and detected *T. gondii* sporozoite mRNA in four of seven mussels. As far as the authors are aware, this is the first time this method has been used to confirm the presence of sporulated *T. gondii* oocysts in shellfish. As it is possible to detect SporoSAG mRNA in sporulated but non-viable oocysts (this study; Villegas et al. 2010; Ware et al. 2010; Travaillé et al. 2016), we cannot definitively claim that infectious oocysts were found in these mussels. Experimental studies using mouse bioassays have shown that shellfish can ingest and retain viable, infectious oocysts (Arkush et al. 2003; Lindsay et al. 2004). Results of the current investigation suggest that this may also occur in shellfish naturally exposed to *T. gondii* and that the infective stage of the parasite could reach consumers.

In conclusion, green-lipped mussels could serve as bio-indicators of coastal protozoal pollution in New Zealand. Further surveillance using wild mussels should aim to characterise the distribution of these potentially pathogenic organisms in the marine environment and identify associated risk factors. This study appears to be the first to demonstrate that naturally-exposed marine shellfish can harbour sporulated oocysts of *T. gondii*, providing an additional molecular tool to evaluate health risks for consumers.

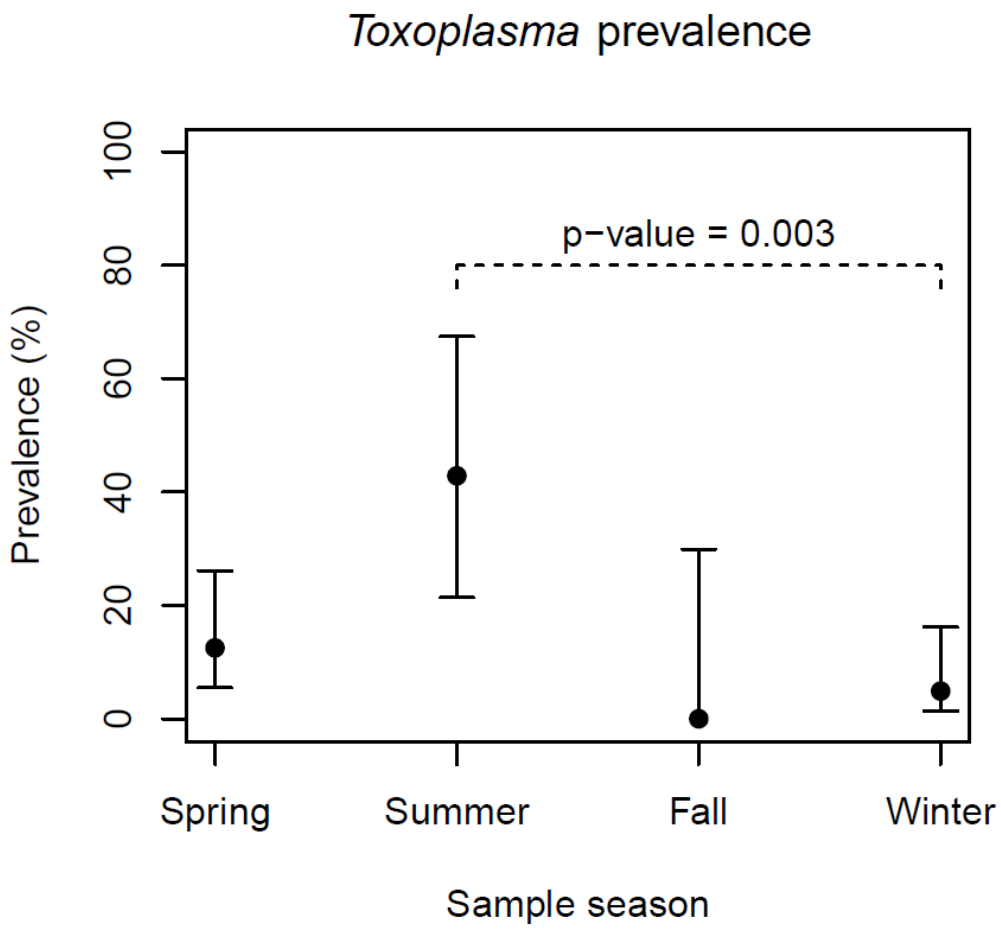
Table and Figure legends

Table 1. Apparent prevalence of *Toxoplasma gondii, Cryptosporidium* and *Giardia duodenalis* DNA in green-lipped mussels (*Perna canaliculus*) collected from eight commercial outlets in New Zealand at seven time points between September 2013 and November 2015. 95 % confidence intervals are given using the Wilson method for binomial proportions. ND = not done

Table 2. Primer sequences, annealing temperatures, and expected amplicon size for the four polymerase chain reaction assays used for detection of *Cryptosporidium* spp. DNA, *Giardia duodenalis* DNA, *Toxoplasma gondii* DNA, and *T. gondii* SporoSAG mRNA in mussel haemolymph

Table 3. Prior information about the diagnostic specificity and sensitivity of a nested polymerase chain reaction assay targeting the *dhps* gene for detection of *T. gondii* DNA in mussel haemolymph was modelled using a beta-distribution elicited from a technical expert. A beta-distribution was also specified for true prevalence of *T. gondii* in mussels informed by overseas prevalence studies

Fig. 1 Seasonal changes in *Toxoplasma gondii* DNA prevalence in green-lipped mussels collected from commercial sources in New Zealand. Univariate analysis showed there was a significantly higher prevalence in mussels sampled in the summer months (December, January, February) compared with winter (June, July, August) ($P = 0.003$). Significance was indicated when $P \leq 0.05$. 95 % confidence intervals are given using the Wilson method for binomial proportions



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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Alicia Coupe
Name/title of Primary Supervisor:	Wendi D. Roe
Name of Research Output and full reference:	
Coupe, A., Howe, L., Burrows, R., Roe, A., Pita, A., Velathanthiri, N., ... & Roe, W. D. (2018). First report of Toxoplasma gondii associated oocysts and Giardia duodenalis in commercial green-lipped mussels (Perna canaliculus) in New Zealand.	
In which Chapter is the Manuscript /Published work:	6
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	75
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Contributed to study design (AC,WR,LH,DH) and sample collection (WR,AS,AC). Conducted laboratory work with T. gondii, aided by LH & EB. Conducted Bayesian analysis &interpreted findings. Wrote manuscript (eds WR,LH,EV,DH,KS)	
For manuscripts intended for publication please indicate target journal:	
Parasitology research, 117(5), 1453-1463	
Candidate's Signature:	Alicia Coupe Digitally signed by Alicia Coupe Date: 2021.01.13 17:03:49 -08'00'
Date:	01/13/21
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Date:	

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

10.11 Appendix 6ii: *Toxoplasma gondii* SporoSAG mRNA RT-PCR

Toxoplasma SporoSAG mRNA RT-PCR

Nucleic acid extraction	**Kit**
RNA	Qiagen RNEasy FFPE Kit

Primers	**Name**	**Sequence (5'-3')**	**Amplicon Size**	**Position***	**Target[1]**
Forward	SporoSAG F	CGG ACA AAT GTG GCG TAC AC	71		mRNA from SporoSAG gene
Reverse	SporoSAG R	GTG ATC TTG CGC CGA ACA C			

* Based on numerical positions of **AY492338.1**

RT-PCR kit: SuperScript® III OneStep RT-PCR System with Platinum *Taq*® DNA Polymerase (Qiagen)

Reagent[2]	**Reaction Volume (25 uL)**
RNase-free water	5.5
2X Reaction Mix	12.5
RT/*Taq* Mix	1.0
10uM SporoSAG F	0.5
10uM SporoSAG R	0.5
mRNA	5.0

! PREPARE ON ICE

PCR controls	**Description**
Positive	Total RNA from 1000 Toxoplasma oocysts
Negative A	Nuclease-free water

PCR Program Name: SporoSAG RT-PCR

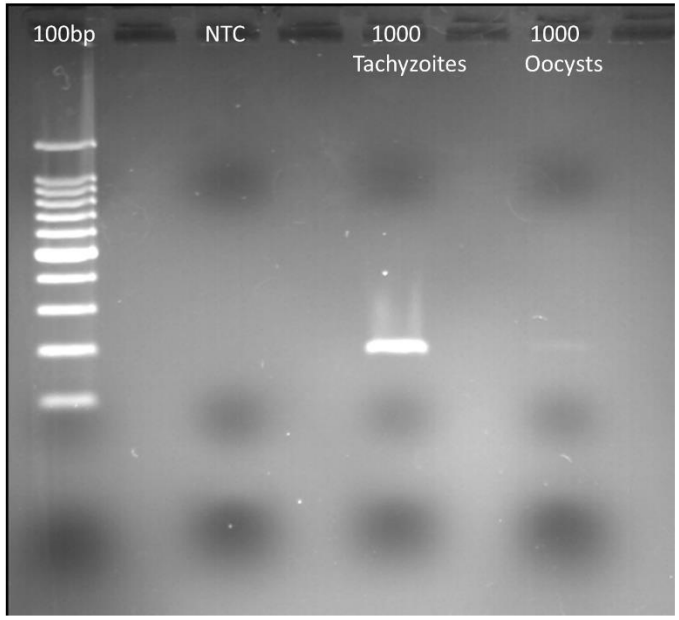
Cycling Parameters[2]	Temperature (°C)	Time	Cycles
RT	55 (pre-heated)	30 min	1
Denaturation	94	2 min	1
Denature	94	15 sec	45
Anneal	60	30 sec	
Extend	68	45 sec	
Final Extension	68	5 min	1
Hold	4	∞	

Electrophoresis	Description	Size of amplicon (bp)
Agarose gel	2.5 %	71
MW marker	20 bp	

Run: 100V for 40 min

References
1. Travaille et al (2016) "Development of a qRT-PCR method to assess the viability of…*Toxoplasma gondii* oocysts", in Food Control (59), pp. 359-365. Note: Authors use the One-Step RT-PCR kit for a real-time assay using same primers but including a fluorescent probe (5'FAM-TTC TCG TCA AAG CGG CAC CAC AGG-3' BHQ1)
2. Qiagen SuperScript® III One-Step RT-PCR Instructions.

SporoSAG RT-PCR using DNA from *T. gondii* tachyzoites and oocysts



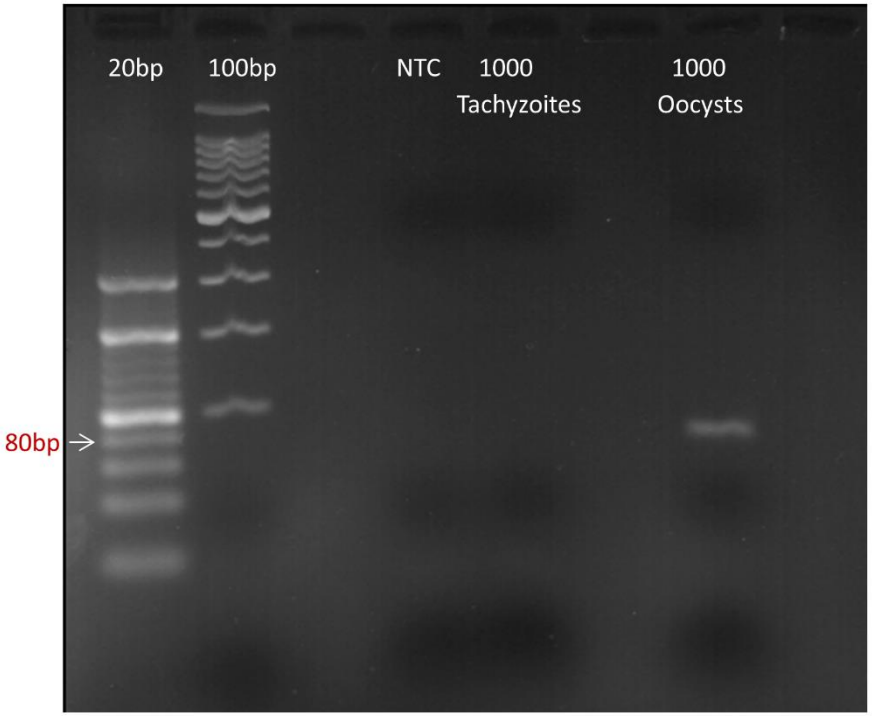
Reagent	25ul rxn
H_2O	18.15
10 x PCR Buffer	2.5
50 mM $MgCl_2$	0.75
dNTPs	0.5
10 mM SporoSAG F	0.5
10 mM SporoSAG R	0.5
Platinum Taq	0.1
DNA	2.0

Cycling Parameters:

Initial Denaturation	95 °C	4 min	
Denature	94 °C	30 s	x 35
Anneal	60 °C	30 s	
Extend	72 °C	1 min	
Hold	72 °C	10 min	
	4 °C	∞	

SporoSAG RT-PCR using RNA from *T. gondii* tachyzoites and oocysts

RT-PCR kit: SuperScript® III OneStep RT-PCR System with Platinum *Taq*® DNA Polymerase (Qiagen)

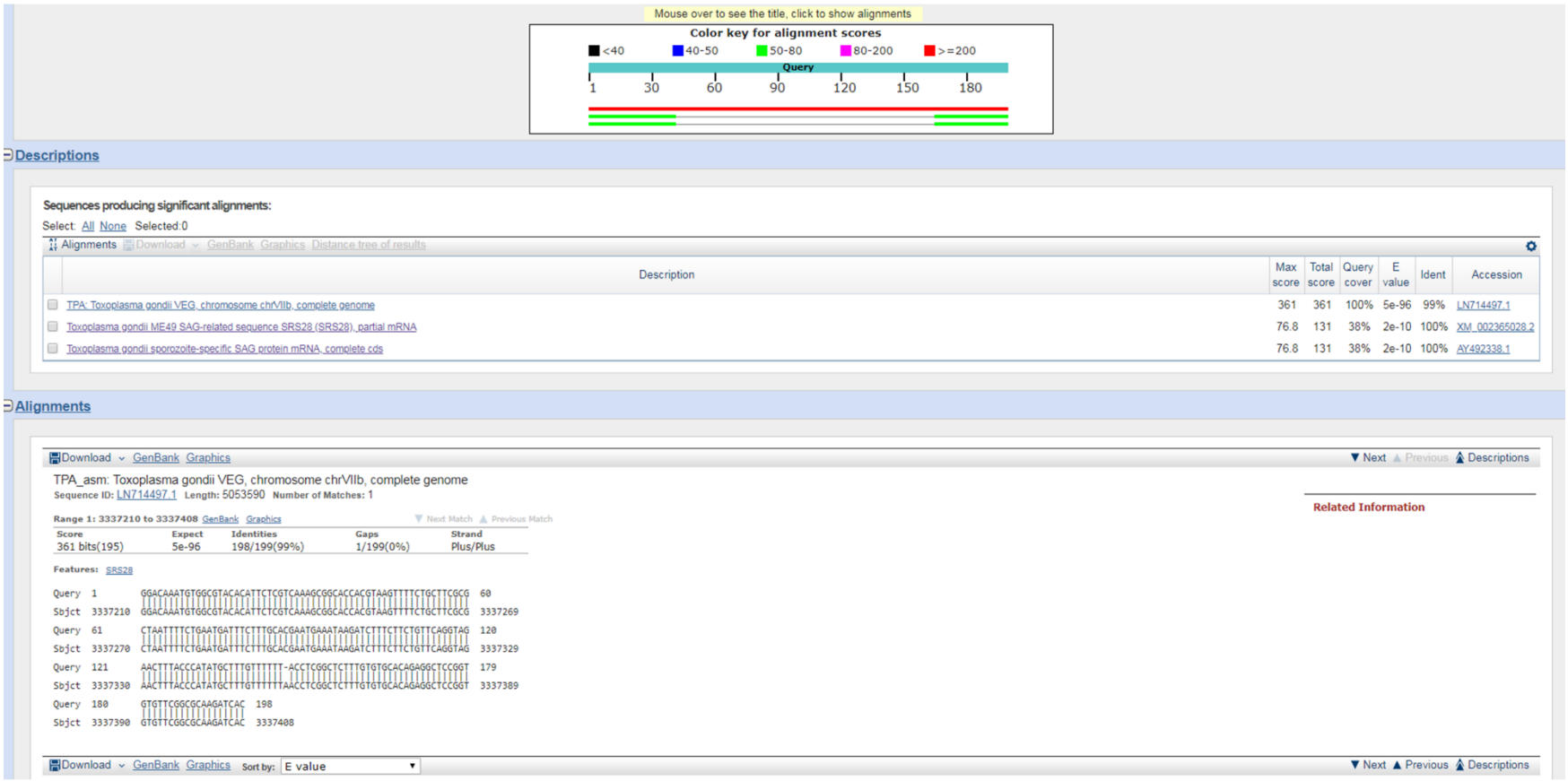


Reagent	25ul rxn
RNase free H_2O	5.5
2X Reaction Mix	12.5
RT/*Taq* Mix	1.0
10 mM SporoSAG F	0.5
10 mM SporoSAG R	0.5
RNA	5.0

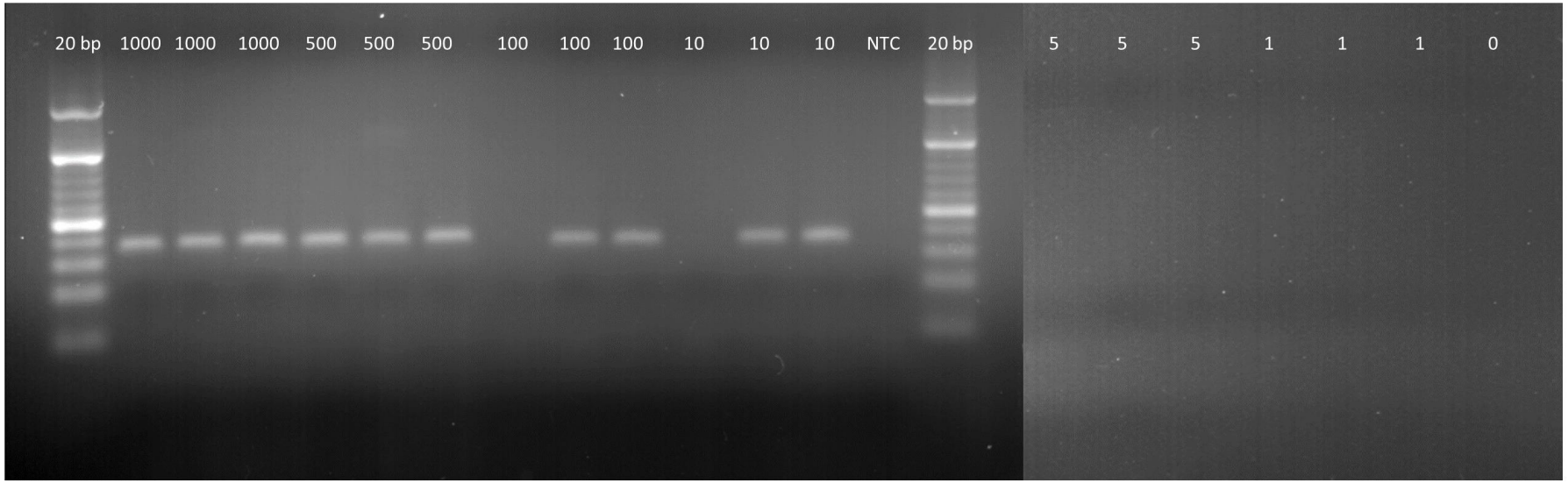
Cycling Parameters:

RT	55 °C	30 min	
Denaturation	94 °C	2 min	
Denature	94 °C	15 s	
Anneal	60 °C	30 s	x 45
Extend	68 °C	45 s	
Final Extension	68 °C	5 min	
Hold	4 °C	∞	

GenBank report of amplicon from SporoSAG RT-PCR using RNA from *T. gondii* oocysts



Sensitivity of SporoSAG RT-PCR using RNA from *T. gondii* oocysts



10.12 Appendix 7i: Coupe et al. (2019)

Comparison of PCR assays to detect *Toxoplasma gondii* oocysts in green-lipped mussels (*Perna canaliculus*)

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Abstract

Toxoplasma gondii is recognised as an important pathogen in the marine environment, with oocysts carried to coastal waters in overland runoff. Currently, there are no standardised methods to detect *T. gondii* directly in seawater to assess the extent of marine ecosystem contamination, but filter-feeding shellfish may serve as biosentinels. A variety of PCR-based methods have been used to confirm presence of *T. gondii* DNA in marine shellfish, however systematic investigations comparing molecular methods are scarce. The primary objective of this study was to evaluate analytical sensitivity and specificity of two nested-PCR (nPCR) assays targeting *dhps* and B1 genes, and two real-time (qPCR) assays targeting the B1 gene and a 529bp repetitive element (rep529), for detection of *T. gondii*. These assays were subsequently validated for *T. gondii* detection in green-lipped mussel (*Perna canaliculus*) haemolymph using oocyst spiking experiments. All assays could reliably detect 50 oocysts spiked into mussel haemolymph. The lowest limit of detection was 5 oocysts using qPCR assays, with the rep529 primers performing best, with good correlation between oocyst concentrations and Cq values, and acceptable efficiency. Assay specificity was evaluated by testing DNA from closely related protozoans, *Hammondia hammondi*, *Neospora caninum*, and *Sarcocystis* spp. Both nPCR assays were specific to *T. gondii*. Both qPCR assays cross-reacted with *Sarcocystis* spp. DNA and the rep529 primers also cross-reacted with *N. caninum* DNA. These studies suggest that the rep529 qPCR assay may be preferable for future mussel studies but direct sequencing is required for definitive confirmation of *T. gondii* DNA detection.

Keywords: *Toxoplasma gondii*; polymerase chain reaction; rep529 repetitive element, B1 gene, *dhps* gene; *Perna canaliculus* green-lipped mussel

Introduction

The zoonotic protozoan, *Toxoplasma gondii*, is a ubiquitous terrestrial pathogen that is also recognised as a waterborne parasite (Dubey 2004; Jones and Dubey 2010; VanWormer et al. 2014). Human and marine mammal infections with *T. gondii*, thought to be associated with exposure to water or prey contaminated with *T. gondii* oocysts, have been reported globally (e.g. Bowie et al., 1997; De Moura et al., 2006; Dubey et al., 2003; Kreuder et al., 2003; Roe et al., 2013). A growing number of studies have used PCR-based methods to detect *T. gondii* DNA/RNA in naturally-exposed freshwater or marine filter-feeding shellfish (e.g. Esmerini et al. 2010; Shapiro et al. 2015; Staggs et al. 2015; Cong et al. 2017; Ghozzi et al. 2017; Coupe et al. 2018), providing further evidence that this parasite is widespread in aquatic environments.

Toxoplasma gondii is the causative agent of toxoplasmosis, which is one of the most common parasitic infections of humans and other warm-blooded animals, including marine wildlife (Tenter et al. 2000; Batz et al. 2012; Dubey 2016; Wilking et al. 2016). Although the majority of *T. gondii* infections are thought to be asymptomatic, toxoplasmosis can have severe consequences for infected hosts, and can be fatal (Mead et al. 1999; Tenter et al. 2000; Holland 2003; Pereira-Chioccola et al. 2009; McLeod et al. 2013). It is thought that *T. gondii* can infect all warm-blooded animals as intermediate hosts, but felids are the only known definitive hosts that can shed oocysts in their faeces (Dubey and Frenkel 1972). Once in the environment, *T. gondii* oocysts sporulate to become infectious, within one to five days of excretion depending upon aeration and temperature (Dubey et al.1998). Sporulated oocysts are extremely hardy, able to survive in soil, freshwater, and saltwater for over a year (Frenkel et al. 1975; Dubey 1998; Lindsay et al. 2003; Lindsay and Dubey 2009), and can pollute freshwater and marine environments, surviving transport to the coast in land-sea runoff (Miller et al. 2002; Conrad et al. 2005; VanWormer et al. 2014). In New Zealand, recent work has shown that *T. gondii* is present in coastal ecosystems (Coupe et al. 2018), and toxoplasmosis has been identified as a cause of mortality for some marine mammal species in the country (Roe et al. 2013, 2017).

Despite the risks that coastal contamination with *T. gondii* oocysts may pose for human and marine wildlife health, at present there are no commercially-available methods to concentrate *T. gondii* oocysts directly from seawater, and molecular confirmation of *T. gondii* in environmental seawater samples has not yet been successful (Jones and Dubey 2010; Shapiro et al. 2010, 2015; Verant et al. 2014). However, filter-feeding shellfish, such as mussels, oysters, and clams, may serve as biosentinels by which to monitor the extent of *T. gondii* pollution in marine ecosystems (Palos Ladeiro et al. 2014; Shapiro et al. 2015; Staggs et al. 2015; Coupe et al. 2018). Studies have shown that shellfish can filter and accumulate sporulated oocysts of *T. gondii* (Arkush et al. 2003; Lindsay et al. 2004; Coupe et al. 2018), and a variety of PCR-based molecular methods have confirmed the presence of *T. gondii* nucleic acids in several naturally-exposed shellfish species (Miller et al. 2008; Esmerini et al. 2010; Putignani et al. 2011; Aksoy et al. 2014; Shapiro et al. 2015; Staggs et al. 2015; Cong et al. 2017; Coupe et al. 2018). Yet, there is no standardised PCR method available for detecting *T. gondii* in shellfish (Hohweyer 2013; Shapiro et al. 2015), and there are only a few studies that compare sensitivity and specificity of these assays for this purpose (Arkush et al. 2003; Putignani et al. 2011; Shapiro et al. 2015; Staggs et al. 2015).

Thus, the primary aim of this study was to evaluate the analytical sensitivity and specificity of four commonly used molecular assays for detection of *T. gondii* tachyzoites and oocysts. Two nPCR assays targeting either the *dhps* (Pashley et al. 1997) or B1 gene (Burg et al. 1989), and two qPCR assays targeting either the B1 gene or a 529 bp repetitive element (Homan et al. 2000) were selected. Additionally, these assays were validated for *T. gondii* detection in green-lipped mussel haemolymph using oocyst spiking experiments to assess their usefulness for *T. gondii* surveillance in coastal ecosystems.

Materials and Methods

Toxoplasma gondii *tachyzoites and oocysts*

Live, attenuated, tachyzoites of Type I S48 strain were obtained from the commercial Toxovax® vaccine (Schering-Plough Animal Health, Wellington, New Zealand) (Hartley and Bridge 1975). *Toxoplasma gondii* oocysts (Type II M4 strain, originally obtained from Lee Innes of the Moredun Research Institute, Edinburgh, Scotland) were provided by Heather Fritz, Jeroen Saeij, and David Arranz Solis (University of California, Davis). Oocysts were not purified but had been heat inactivated by immersion in an 80 °C dry bath for 20 min.

Toxoplasma gondii *stock preparation*

The concentration of stock solutions of tachyzoites and oocysts was determined using a haemocytometer chamber and light or epifluorescence microscopy, respectively. Suspensions of 1000, 100, 10, 1 tachyzoites and 1000, 100, 50, 10, 5, 1 sporulated oocysts were then prepared by serial dilution with sterile phosphate-buffered saline (PBS) (1X, pH 7.4) and used as PCR controls and to evaluate the analytical sensitivity of the PCR assays. Samples were centrifuged at 20,000 ×*g* for 10 min, the supernatant removed, and the cell pellet suspended in 100 µl PBS (1×, pH 7.4).

Haemolymph spiking with* T. gondii *oocysts

Haemolymph was obtained from commercial green-lipped mussels previously determined to be negative for *T. gondii* DNA in duplicate PCR tests. Hemolymph from several mussels was pooled and divided into 100 µl aliquots. Each 100 µl aliquot was spiked with serial dilutions of *T. gondii* oocysts before the haemolymph was pelleted, supernatant aspirated, and cell pellet suspended, as above.

DNA Extraction

DNA was extracted from serially diluted tachyzoites, oocysts, and oocyst-spiked haemolymph, using Qiagen DNeasy Blood and Tissue kits® (Qiagen, Hilden, Germany). For tachyzoites, DNA was extracted according to the manufacturer's instructions for non-nucleated blood, including an overnight digest at 56 °C with proteinase K. . For oocysts and oocyst-spiked haemolymph, DNA was extracted following

procedure detailed in Shapiro et al. (2015). Briefly, ATL buffer (180 µl) was added to each sample before one freeze-thaw cycle of immersion in liquid nitrogen (4 min)/boiling water (4 min). Proteinase K (40 µl) was then added before incubation overnight at 56 °C. After addition of Buffer AL (200 µl), samples were incubated at 70 °C for 10 min. DNA was eluted in 10 % AE solution (50 µl), which was first heated to 95 °C. After application of elution buffer, spin columns were incubated at room temperature for 5 min before being centrifuged (8,000 x*g*, 2 min). A non-spiked haemolymph and PBS only samples were included to serve as additional negative controls in the DNA extractions and subsequent PCR reactions. DNA was extracted for each parasite concentration and stored at -80 °C until molecular analysis.

PCR protocols

nPCR protocols

Two nPCR assays were evaluated, one using primers targeting the dihydropteroate synthase (*dhps*) gene (Pashley et al. 1997), and the other targeting the B1 gene (Burg et al. 1989). The nPCR *dhps* assay targeted a 450 bp fragment of the *T. gondii dhps* gene using FOOD1/2 and FOOD3/4 primers (Aspinall et al. 2002), as described by Roe et al. (2013) (Table 1). For detection of *T. gondii* oocyst DNA, bovine serum albumin (BSA, 10 mg/ml) was added to each PCR reaction mix to minimise effects of PCR inhibitors. The nPCR B1 targeted a 530 bp fragment of the *T. gondii* B1 gene using Pml/S1, Pml/AS1, Pml/S2, and Pml/AS2 primers, as described by Grigg and Boothroyd (2001), with the addition of BSA (1 mg/ml) to each PCR reaction mix (Shapiro et al. 2015) (Table 1). All PCR reactions were performed using a conventional PCR thermal cycler (Veriti 96 Well Thermal Cycler, Applied Biosystems Inc, CA, USA) and each PCR assay was run in triplicate per parasite concentration. To confirm successful amplification, 10 µl of the final PCR product was run on a 1.5 % agarose gel stained with SYBR Safe (Thermo Fisher Scientific), before visualisation by UV light using an E-Gel Imager (Life Technologies, Carlsbad, CA, USA). Presence of a band of expected size, consistent with that of the positive control, was taken as qualitative evidence of successful amplification of *T. gondii* DNA. The size of the PCR amplicon was estimated by comparison with a 100 bp DNA ladder (Promega, Madison WI, USA). Controls included DNA extracted from a known *T. gondii* isolate (incomplete strain S48, Toxovax®) and ultrapure water as a negative control. Assay specificity was verified

using DNA extracted from closely related protozoans, *Hammondia hammondi*, *Neospora caninum* and *Sarcocystis* spp., whose identities had been previously confirmed by sequencing. *Hammondia hammondi* DNA was available from a feline faecal float with microscopically-observed *Hammondia*-like oocysts.

Sarcocystis spp. DNA was available from alpaca skeletal tissue with observable *Sarcocystis* spp. bradyzoite stages. *Neospora caninum* DNA was available from cell cultured *N. caninum* tachyzoites, originally sourced from a calf brain (Okeoma et al. 2004).

qPCR protocols

Two genomic targets were compared using qPCR, the B1 gene and the 529 bp repeat element (rep529) (Homan et al. 2000). Primers used for the qPCR B1 were oligo1 and oligo4, as described by Burg et al. (1989), producing a target amplicon of 193 bp (Table 1). Primers used for the rep529 qPCR were ToxoRE_f and ToxoRE_r, as described by Kasper et al. (2009), with slight modifications, producing a target amplicon of 81 bp (Table 1). Probes were not included in either qPCR assay. Targets were amplified using FastStart Universal SYBR Green Master (ROX) (Roche, Manheim, Germany) two-step method. Final reaction mixtures for tachyzoite experiments (20 µl total) included 10 µl 2X FastStart Universal SYBR Green Master (ROX), 0.25 µM forward primer, 0.25 µM reverse primer, and 2 µl of template DNA. Final reaction mixtures for oocyst experiments (10 µl total) included 5 µl 2X FastStart Universal SYBR Green Master (ROX), 0.25 µM forward primer, 0.25 µM reverse primer, 0.5 µl BSA (10 mg/mL), and 2 µl of template DNA. A Mic qPCR cycler (Bio Molecular Systems, Queensland, Australia) was used for all qPCR analyses. Amplification conditions were 95 °C 15 min followed by 40 cycles at 95 °C for 15 sec, 53 °C for 30 sec (B1) or 54 °C for 30 sec (rep529), and 72 °C for 30 sec. Each reaction was followed by a melting curve 70 to 85 °C, 0.1 °C per sec. Standard curves and reaction efficiencies were calculated for each run using micPCR version 2.6.4 (Bio Molecular Systems). A run was considered valid if the qPCR efficiency was > 90 %. Correlation between parasite concentration and Cq values was considered to be 'poor' if the R^2 value < 0.90, 'fairly good' if $0.90 \leq R^2 < 0.95$, 'good' if $0.95 \leq R^2 < 0.98$, and 'excellent' if $R^2 \geq 0.98$. Assay specificity was further verified using DNA extracted from closely related

protozoans, as described above. Positive and negative controls as described above were also included for all qPCR runs.

Results

Comparison of PCR performance using DNA from T. gondii tachyzoites

Sensitivities of the four assays were initially assessed using DNA extracted from known quantities of tachyzoites. Tachyzoites were used for initial assay optimisation as they were readily available, whereas oocysts were not immediately available due to the need for animal infection experiments for their production. All four PCR assays were able to detect DNA from 1000 to 10 tachyzoites, but the nPCR B1 was most sensitive, able to consistently detect DNA from a single tachyzoite (Table 2). The limit of detection of the nPCR *dhps* assay was found to be 10 tachyzoites. Sensitivity was determined to be 66.7 % for 10 *T. gondii* tachyzoites (2/3 PCR replicates) and 100 % for 100 and 1000 tachyzoites (3/3 PCR replicates), whereas the limit of detection of the nPCR B1 was 1 tachyzoite (3/3 replicates). For both qPCR assays, there was good correlation ($R^2 = 0.97$) between Cq values and tachyzoite concentration, although the limit of detection for both assays was higher than the B1 nPCR, at 10 tachyzoites (3/3 replicates) (Table 2). Melt curve analysis revealed that PCR products from the qPCR B1 had melting temperatures of 79.7 ± 0.2 °C (Fig. 1A). The qPCR B1 did amplify DNA from a single tachyzoite (1/3 replicates) but the melting temperature of this product was outside of the predicted temperature range, at 78.3 °C, and therefore was considered non-specific. Melt curve analysis of the rep529 qPCR assay showed a single peak between 80.0 ± 0.1 °C for all amplicons from tachyzoites (Fig. 1B).

Comparison of PCR performance using DNA from* T. gondii *oocysts

Sensitivities of the four assays were further assessed using DNA extracted from known quantities of *T. gondii* oocysts (Table 2). Although sensitivities varied between the assays, all could consistently detect

DNA extracted from 50 – 1000 oocysts. The rep529 qPCR was most sensitive, able to consistently detect DNA from as few as 5 oocysts. The nPCR *dhps* could only reliably detect 50 oocysts or higher (3/3 replicates). However, a lower limit of detection was achieved with the nPCR B1, which had a sensitivity of 66.7 % for 10 oocysts (2/3 replicates) and 5 oocysts (2/3 replicates). The qPCR B1 could also only reliably detect 50 oocysts (3/3 replicates), with a sensitivity of 33.3 % for 10 and 5 oocysts (1/3 replicates) (Table 2). Although there was good agreement (R^2 = 0.97) between Cq values and oocyst dilutions for the qPCR B1, PCR efficiency was poor, only reaching 70 %. Most qPCR B1 amplicons had consistent melting temperatures of 80.1 ± 0.2 °C which was a slight shift to the right from the melting temperatures observed with the tachyzoite amplicons (Fig. 1A). The rep529 qPCR assay was most sensitive, amplifying all three replicates of 5 oocysts and above (Table 2). There was good agreement (R^2 = 0.97) between Cq values and oocyst dilutions for this assay, with a single melt curve peak between 80.0 ± 0.1 °C (Fig. 1B).

Comparison of PCR performance using DNA from haemolymph spiked with T. gondii oocysts

Finally, the four PCR assays were evaluated for the detection of DNA from *T. gondii* oocysts spiked into green-lipped mussel haemolymph (Table 3). All four assays consistently detected 50 oocysts spiked into mussel haemolymph (3/3 replicates). The qPCR assays were most sensitive, with a limit of detection of 5 oocysts, whereas both nPCR assays failed to amplify DNA at lower oocyst concentrations. Although the qPCR B1 was relatively sensitive, agreement between Cq values and number of spiked oocysts was poor (R^2 = 0.73), and efficiency was excessively high (147 %) when detecting *T. gondii* DNA from 10 and 5 spiked oocysts for a single replicate. Melt curves for qPCR B1 amplicons from 50 spiked oocysts or higher produced peaks between 80.0 ± 0.1 °C (Fig. 1A). For amplicons from less than 10 spiked oocysts, only the non-specific melt curve peak was observed. The rep529 qPCR assay was able to detect DNA from 10 and 5 spiked oocysts for a single replicate with good agreement (R^2 = 0.97) and consistent melt curve peaks between 79.9 ± 0.1 °C (Fig. 1B), although PCR efficiency was reduced to 82 %.

Evaluation of primer specificity

When specificity of the protocols was tested against other apicomplexan parasites, the nPCR assays were the most specific, detecting only *T. gondii* DNA, while both qPCR assays cross-reacted with *Sarcocystis* spp. The rep529 qPCR also cross-reacted with *Neospora caninum* (Fig. 1D). The qPCR B1 amplified *Hammondia hammondi* and *Neospora caninum* DNA but the PCR products had melting temperature peaks at 81.8 °C and 78.1 °C, respectively (Fig. 1C), and so could be distinguished from peaks generated from *T. gondii* target DNA.

Discussion

Due to a lack of efficient and standardised methods for direct detection of *T. gondii* oocysts in seawater, testing of filter-feeding shellfish as biosentinels of aquatic ecosystem pollution has been advocated as an alternative surveillance strategy (Palos Ladeiro et al. 2014; Shapiro et al. 2015; Staggs et al. 2015; Kerambrun et al. 2016; Coupe et al. 2018). Many molecular assays have been developed for the specific detection of *T. gondii* in biological samples (Su et al. 2010; Bahia-Oliviera et al. 2017) and several have now been adapted for use in shellfish, however method standardisation is scarce (Shapiro et al. 2015; Bahia-Oliviera et al. 2017). The B1 gene is the most widely used PCR target in shellfish studies (Arkush et al. 2003; Esmerini et al. 2010; Putignani et al. 2011; Aksoy et al. 2014; Marangi et al. 2015; Marquis et al. 2015; Shapiro et al. 2015; Cong et al. 2017; Ghozzi et al. 2017), followed by the rep529 marker (Palos Ladeiro et al. 2014; Ribeiro et al. 2015; Staggs et al. 2015; Kerambrun et al. 2016), in both conventional and qPCR assays. Other PCR targets include the 18S rRNA (Arkush et al. 2003; Miller et al. 2008), ITS-1 (Zhang et al. 2014; Shapiro et al. 2015), SAG1 (Ribeiro et al. 2015; Shapiro et al. 2015), and *dhps* (Coupe et al. 2018; this study) genes. Despite the variety, there appears to be a consensus in the *T. gondii* literature that PCR assays based upon the rep529 marker generally perform best in terms of sensitivity, independently of the DNA primers and PCR technology used, and also sample type (Edvinsson et al. 2006; Kasper et al. 2009; Yang et al. 2009; Sterkers et al. 2010; Su et al. 2010; Staggs et al. 2015; Wells et al. 2015). Indeed, the rep529 qPCR assay described by Kasper et al. (2009) yielded a sensitivity of 1/30 to 1/50 of a single parasite genome (assuming there are 200 – 300

copies of the rep529 marker in the *T. gondii* genome) per PCR reaction, determined using a plasmid standard dilution series. With respect to shellfish, few prior studies have used oocyst spiking experiments to assess PCR performance (Esmerini et al. 2010; Shapiro et al. 2015; Staggs et al. 2015), and authors report varied sensitivities. Using a nPCR assay targeting the B1 gene, Esmerini et al. (2010) reported detection limits of 1000 and 100 oocysts in tissue homogenates from mussels and oysters, respectively. A detection limit of 5 oocysts in mussel haemolymph was achieved by Shapiro et al. (2015), also using a nPCR assay targeting the B1 gene, as well with nPCR assays targeting the ITS-1 gene, and the rep529 marker. Both the rep529 conventional and qPCR assays reported by Staggs et al. (2015) could consistently detect a single oocyst spiked into mussel haemolymph, currently representing the most sensitive molecular assays described in shellfish.

In the present study, the performance of four previously published PCR assays was compared using *T. gondii* tachyzoites, oocysts, and oocyst-mussel spiking experiments. The primary aim of the study was to determine the best-performing assay for detection of *T. gondii* oocyst DNA in the green-lipped mussel, as a potential bioindicator of *T. gondii* pollution in marine ecosystems in New Zealand. Spiking experiments showed that the rep529 qPCR provided the best sensitivity for detection of *T. gondii* oocyst DNA in the green-lipped mussel, with good correlation between oocyst concentrations and Cq values and a detection limit of 5 spiked oocysts, comparable to previous spiking studies (Shapiro et al. 2015). Although the qPCR B1 provided a detection limit of 5 spiked oocysts, this assay performed poorly in terms of efficiency, and the correlation between oocysts numbers and Cq values was low. Non-specific amplification was also observed, even after assay optimisation, so its use for detection of *T. gondii* oocysts in shellfish is probably limited. Both nPCR assays consistently detected 50 spiked oocysts, but not fewer. Spiking experiment results, therefore, appear to support previous findings that the rep529 target offers a sensitive tool for detection of *T. gondii* oocyst DNA in shellfish (Staggs et al. 2015).

Interestingly, results demonstrated that the nPCR B1 proved to be most sensitive when the assay was evaluated using tachyzoites, with amplification of DNA from a single parasite, compared with the

rep529 qPCR (and other assays) which amplified DNA from 10 tachyzoites. While oocysts are the hardy life-stage of *T. gondii* that can survive in the environment and accumulate in shellfish, it is the tachyzoite and bradyzoite (in tissue cysts) life stages that are found in tissues of infected warm-blooded hosts. At present, the nPCR *dhps* evaluated in this study is favoured for molecular confirmation of toxoplasmosis in infected warm-blooded hosts in New Zealand (Roe et al. 2013, 2017; Howe et al. 2014; Patel 2016). Results suggest that, although the rep529 qPCR performed best to detect *T. gondii* oocysts in green-lipped mussels, the nPCR B1 may be preferable for testing infected intermediate hosts due to its superior sensitivity using tachyzoite DNA. This assay is also dual-purpose, as the B1 locus can be used for genotyping (Grigg and Boothroyd 2001; Shapiro et al. 2015).

The rep529 qPCR was considered to be the most sensitive assay for testing green-lipped mussels in this study. The increased sensitivity of rep529-based PCR assays could be due to differences in PCR target copy numbers. Both the rep529 and the B1 gene are multi-copy, but there are up to 20 times as many copies of the rep529 marker in the *T. gondii* genome, compared to the B1 (Burg et al. 1989; Homan et al. 2000; Reischl et al. 2003; Costa and Bretagne 2012), while the copy number of the *dhps* gene remains to be determined. The sensitivity of the rep529 qPCR, however, cannot fully be explained by differences in PCR target copy number, as the nPCR B1 provided the best sensitivity for detection of tachyzoite DNA, and was also comparable to the rep529 qPCR for detection of DNA from free oocysts. Results of the present study highlight the importance of using oocyst spiking experiments in determining assay sensitivity, particularly because of the presence of PCR inhibitors (Staggs et al. 2015; Kerambrun et al. 2016), which in shellfish tissues can include glycogen and acidic polysaccharides (Schwab et al. 1998). Haemolymph seems to be the least inhibitory tissue of shellfish for molecular testing, as it is a less dense and complex matrix than gill or digestive gland (Esmerini et al. 2010; Palos Ladeiro et al. 2015; Shapiro et al. 2015; Staggs et al. 2015). Nevertheless, all four assays evaluated in this study performed better when applied on DNA from tachyzoites and free oocysts in PBS as compared with oocysts spiked into mussel haemolymph, despite the addition of BSA to PCR reactions (Jiang et al. 2005). Results showed that when applied on oocysts-spiked in haemolymph, the assays suffered reduced sensitivity, or reduced qPCR assay efficiency/R^2 values, or both. Particularly, the

sensitivity of the nPCR B1 was reduced 10-fold from 5 to 50 oocysts when oocysts were spiked into haemolymph. The traditional approach for determining assay sensitivity has been to use plasmids or dilution series of genomic DNA from tachyzoites (Staggs et al. 2015). Yet, results indicate that if we had taken this approach, we would have concluded that the nPCR B1 performed better than the rep529 qPCR, whereas the nPCR B1 seemed to be particularly affected by PCR inhibitors specific to green-lipped mussel haemolymph in this study. Use of the nPCR B1 assay in shellfish surveillance studies in New Zealand may therefore underestimate *T. gondii* prevalence, although further research is needed.

The use of oocysts to validate methods for *T. gondii* detection in environmental samples is also important because a single sporulated oocyst contains eight individual parasites (sporozoites), i.e. eight *T. gondii* genomes, whereas one tachyzoite contains a single genome (Dubey et al. 1998), so comparison of PCR assay sensitivities between studies using plasmids or different life-stages of *T. gondii* may be misleading. Furthermore, the oocyst life-stage is particularly resistant to environmental stressors, requiring freeze-thaw cycles or bead-beating to fracture the tough oocyst wall (Hohweyer 2013), which may impact DNA extraction, and subsequent molecular analyses.

While the rep529bp assay appeared to perform well compared with other assays previously evaluated in shellfish, it is difficult to compare assay performance across studies. This is not only because authors use different PCR targets, primers, and amplification conditions, but also because of protocol variations that may influence detection limits, such as the type of standard samples used and shellfish tissue tested. The application of different DNA/RNA extraction methods will further influence PCR detection limits (Bastien et al. 2008), as well as differences in inhibitor removal abilities between preparations (Staggs et al. 2015). For example, the rep529-based PCR assay described by Staggs et al. (2015) are reportedly the most sensitive assays overall for testing shellfish for *T. gondii*, as they were able to consistently detect a single spiked oocyst. The increased sensitivity achieved by Staggs et al., may be due to the fact that oocysts used here were not purified, to better mimic natural conditions, and because the detection limit was based on the serial dilution of a haemocytometer-counted suspension,

which may be less accurate than fluorescence activated cell sorting. However, it may also be due to differences in DNA extraction methods between studies. Notably, the Staggs protocol spiked oocysts into haemolymph samples after the haemolymph had been pelleted, whereas in this study (following Shapiro et al. 2015), oocysts were spiked into haemolymph samples before they were pelleted, again to better mimic natural conditions. Sensitivity was reduced to 10 oocysts for Staggs et al. when results were re-evaluated using an alternative DNA extraction protocol. In summary, there may be tissue- and possibly species-specific PCR inhibitors present in shellfish which could affect PCR performance. Therefore, spiking experiments are required to evaluate matrix-specific inhibition, as well as protocols for preparation of nucleic acids prior to PCR, taking into account that there may be a trade-off between sensitivity and removal of PCR inhibitors (Schrader et al. 2012; Staggs et al. 2015).

Maximum sensitivity is desired for *T. gondii* detection in shellfish because there are likely to be low oocyst numbers in shellfish tissues (< 100 parasites per mussel) (Hohweyer 2013; Aksoy et al. 2014; Marangi et al. 2015). However, it is also important that PCR assays are specific, to minimise false positive results. Previous work claims that the highly sensitive rep529 marker is also highly specific to *T. gondii* (Homan et al. 2000; Kasper et al. 2009), but results of the present study indicate that this is not always the case. Particularly, we found that the rep529 primers (Kasper et al. 2009) evaluated in this study cross-reacted with *Sarcocystis* spp. and *N. caninum* DNA, both protozoans that are closely related to *T. gondii*, which may also contaminate marine ecosystems (Dubey et al. 2003; Miller et al. 2010; Michaels et al. 2016). Results suggest that it may be necessary to incorporate the associated probe of Kasper et al. (2009) (ToxoRE_p (FAM-5'-CTA CAG ACG CGA TGC C-3'-NFQ-MGB; FAM, 6-carboxy-fluorescin; NFQ-MGB, nonfluorescent quencher plus attached MGB), although specificity of the primer and probe set was not assessed by the authors using DNA from related protozoans. Kasper et al. (2009) also reported using a higher annealing temperature (62 °C) to ensure 100 % specificity, determined using the primer pair in a SYBR Green qPCR assay. However, in the present study an annealing temperature of 62 °C gave rise to dual bands on conventional temperature gradient PCR, and dual melt curve peaks on qPCR, with a weaker band or smaller peak at the expected amplicon size or melt temperature, respectively, a phenomenon also observed by Yang et al. (2009) (using a different set of

rep529 primers, from Reischl et al. (2003)). An annealing temperature of 54 °C was optimal for the rep529 primers used in this study. Results also confirm that false positive amplification can occur with other common PCR targets, as the qPCR B1 cross-reacted with *Sarcocystis* spp. DNA. A recent study by Shapiro et al. (2015) found that ITS-1 and B1 primer sets generated a high proportion of false positives, due to the presence of DNA from mussels, amoeba, algae, phytoplankton, as well as other related protozoans. Therefore, the rep529 qPCR in this study may serve as a sensitive screening assay for use with green-lipped mussels, but sequence analysis will be required for definitive confirmation of *T. gondii* DNA (Shapiro et al. 2015; Bahia-Oliviera et al. 2017).

In conclusion, the rep529 qPCR used in this study was found to be a suitably sensitive assay to detect the low numbers of oocysts expected in naturally-exposed shellfish. However, an important finding drawn from the study is that primers/PCR targets thought to be highly specific to *T. gondii*, including the rep529 marker, may in fact cross amplify non-target organisms, confirming the importance of direct sequencing of PCR products as a confirmatory test. Particularly, this study confirmed that oocyst spiking experiments are a vital component of PCR validation and assay comparison, as assays may be impacted to varying degrees by inhibitors present in shellfish tissues, which will affect prevalence estimates in surveillance studies. In summary, assays for detection of *T. gondii* in environmental matrices should be carefully selected based on study aims, targeted parasite life-stage, and sample type to be tested, and guided by assay validation procedures for specific experimental conditions and matrix types.

Table 1 Primer sequences, annealing temperatures, and expected amplicon size for the four polymerase chain reaction assays used for detection of *Toxoplasma gondii* DNA

PCR Type	Gene Target	Primer Name	Primer Sequence 5' – 3'	Annealing Temp (°C)	Size (bp)	Reference
nPCR	dhps	FOOD1	GGA ACA TCC GCT GAA GCT CAT GG	57	494	Aspinall et al. (2002)
		FOOD2	CAG AGA ATC CAG TTG TTT CGA GG	57	450	
		FOOD3	CAG TCC AGA CTC GTT CAC CGA TC			
		FOOD4	CCG GAA TAG TGA TAT ACT TGT AG			
	B1	Pml/S1	TGT TCT GTC CTA TCG CAA CG	60	579	Grigg and Boothroyd (2001)
		Pml/AS1	TCT TCC CAG ACG TGG ATT TC			
		Pml/S2	ACG GAT GCA GTT CCT TTC TG	60	530	
		Pml/AS2	CTC GAC AAT ACG CTG CTT GA			
qPCR	B1	oligo1	GGA ACT GCA TCC GTT CAT GAG	53	193	Burg et al. (1989)
		oligo4	TCT TTA AAG CGT TCG TGG TC			
	529 bp Repeat Element (rep529)	ToxoRE_f	CAC AGA AGG GAC AGA AGT CG	54	81	Kasper et al. (2009)
		ToxoRE_r	CAG TCC TGA TAT CTC TCC TCC AAG			

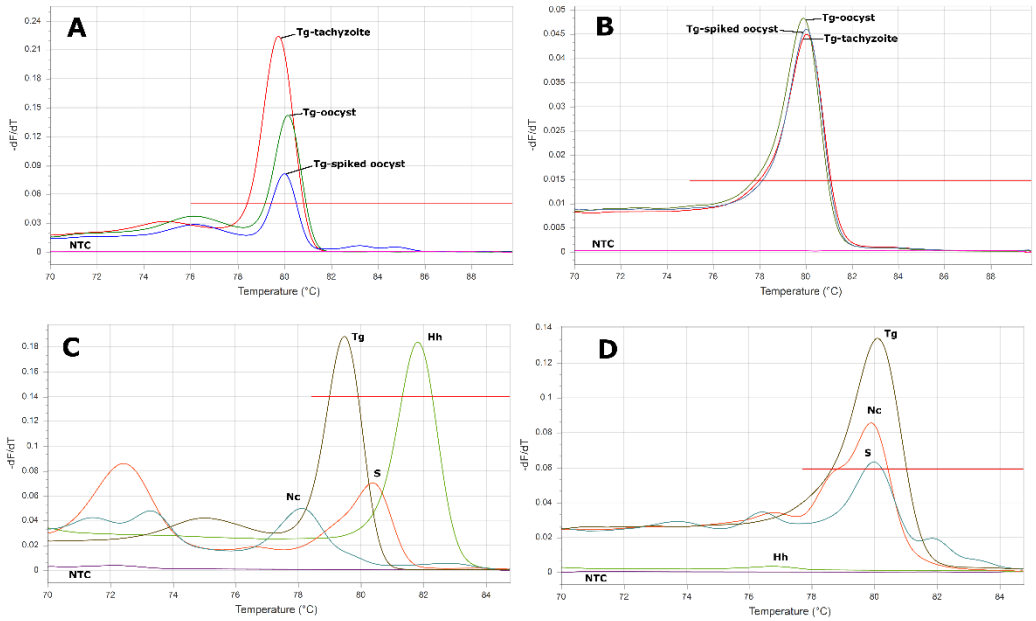
Table 2 Sensitivity of four PCR methods for detection of *Toxoplasma gondii* DNA from known quantities of *T. gondii* tachyzoites and oocysts

	nPCR		qPCR	
No. tachyzoites	***dhps***	**B1**	**B1**	**RE**
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
10	2/3	3/3	3/3	3/3
1	0/3	3/3	0/3	0/3
0	0/3	0/3	0/3	0/3
No. oocysts	***dhps***	**B1**	**B1**	**RE**
1000	3/3	3/3	3/3	3/3
100	2/3	3/3	3/3	3/3
50	3/3	3/3	3/3	3/3
10	0/3	2/3	1/3	3/3
5	0/3	2/3	1/3	3/3
1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

Table 3 Sensitivity of four PCR methods for detection of *Toxoplasma gondii* DNA from known quantities of *T. gondii* oocysts spiked into mussel haemolymph

	nPCR		qPCR	
No. oocysts	***dhps***	**B1**	**B1**	**RE**
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
50	2/3	3/3	3/3	3/3
10	0/3	0/3	1/3	1/3
5	0/3	0/3	1/3	1/3
1	0/3	0/3	0/3	0/3
0	-	-	-	-

Fig. 1 Melt curves from qPCR amplification of the B1 gene (**A**, **C**) and rep529 marker (**B**, **D**) of *Toxoplasma gondii*. **A**, **B** using DNA extracted from known quantities of tachyzoites (Tg-tachyzoite), oocysts (Tg-oocyst), and oocyst-spiked green-lipped mussel (*Perna canaliculus*) haemolymph (Tg-spiked oocyst). **C**, **D** using DNA extracted from related protozoa, previously confirmed by sequencing. Tg, *Toxoplasma gondii* positive control; NTC, no template control (ultrapure water); Hh, *Hammondia hammondi*; Nc, *Neospora caninum*; S, *Sarcocystis* spp.



On behalf of all authors, the corresponding author states that there is no conflict of interest

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Alicia Coupe
Name/title of Primary Supervisor:	Wendi D. Roe
Name of Research Output and full reference:	
Coupe, A., Howe, L., Shapiro, K., & Roe, W. D. (2019). Comparison of PCR assays to detect Toxoplasma gondii oocysts in green-lipped mussels (Perna canaliculus).	
In which Chapter is the Manuscript /Published work:	7
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Contributed to study design (AC,WR,LH) & oocyst preparation (KS,AC). Conducted laboratory work, aided by LH,KS. Conducted melt curve analysis with LH. Wrote manuscript (eds LH,KS,WR).	
For manuscripts intended for publication please indicate target journal:	
Parasitology research, 118(8), 2389-2398.	
Candidate's Signature:	Alicia Coupe Digitally signed by Alicia Coupe Date: 2021.01.13 17:03:49 -08'00'
Date:	01/13/21
Primary Supervisor's Signature:	Wendi Roe Digitally signed by Wendi Roe Date: 2021.01.22 07:40:44 +13'00'
Date:	

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

10.13 Appendix 7ii: *Toxoplasma gondii* qPCR B1 gene

Toxoplasma B1 qPCR

Nucleic acid extraction	**Kit**
DNA	Qiagen DNEasy Blood and Tissue Kit

Primers	**Name**	**Sequence (5'-3')**	**Amplicon Size**	**Position**	**Target[1]**
Forward	B1S	GGA ACT GCA TCC GTT CAT GAG	~200 bp	81	Fragment of B1
Reverse	B1AS	TCT TTA AAG CGT TCG TGG TC		161	

qPCR kit: FastStart Universal SYBR Green Master

Reagent[2]	**Reaction Volume (20 uL)**
SYBR Green Master	10.0
PCR grade water	7.6
25uM oligo1 (B1S)	0.2
25uM oligo 4 (B1AS)	0.2
Template DNA	2.0

PCR controls	Description
Positive	Toxoplasma DNA extracted from Toxovax
Negative	Nuclease-free water

PCR Program Name: Toxo B1 qPCR

Cycling Parameters[2]	Temperature (^{o}C)	Time	Cycles
	95	10 min	1
	95	15 sec	40
	53	30 sec	
	72	30 sec	

References
1. Burg et al. 1989 (oligo 1 and oligo 4)
2. FastStart Universal SYBR Green Master (ROX) Instructions (2008) Version 04

10.14 Appendix 8: Mussel collection dates

Mussel collection dates with high and low risk sampling dates determined by calculating and assessing cumulative rainfall (mm) in the 30 days before mussel collection

date	raglan	day	week	2week	3week	month	cumulative rainfall (mm) in preceding...
4/11/2014	6.5	15.25	16.5	23.75	25.25	47.25	
5/13/2014	4	0	31.5	53	81.5	159.5	
7/10/2014	0.5	0.5	8.25	88.5	119.75	277.5	
12/21/2014	0.75	5.75	49.5	73	78	114	
2/14/2015	0	0	0.75	29.5	29.5	31	low risk
4/6/2015	0	3	4	24.75	29.5	54	
7/15/2015	18.75	0	2	66.25	76.5	96.25	
10/10/2015	0	0	7.75	24.75	68.25	113	
3/9/2016	0.5	0.5	0.5	42.25	82.5	83	
7/1/2016	0	9.25	107.5	146.75	168	169.5	high risk
1/21/2017	13.5	4.75	30	45	68.75	88.25	

date	kaawa	day	week	2week	3week	month	cumulative rainfall (mm) in preceding...
7/11/2014	0	0	7	66.5	98	147.5	
7/12/2014	26	0	5	58.5	98	120	
10/1/2014	0	0.5	8.5	70.5	107	163	
6/13/2015	0	0.5	11.5	84	154	207.5	
10/9/2015	0	0.5	14.5	19	41.5	68.5	
3/10/2016	0	0	0	60	73	101.5	
7/2/2016	0	0	96	144	155	164.5	
1/20/2017	3.5	6	19	20	34	52	