The *Eimeria* species affecting brown kiwi; host-parasite interactions and conservation implications

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Unless someone like you cares a whole awful lot,

Nothing is going to get better. It's not.

- The Lorax, Dr Seuss

To Dez,

Kwa heri, rafiki. Safari salama.

(Good bye, my friend. Safe journey.)

Abstract

Brown kiwi (*Apteryx mantelli*) are a threatened flightless nocturnal ratite endemic to New Zealand. The conservation of this species currently utilises a recovery programme known as 'Operation Nest Egg' (ONE) to increase numbers of brown kiwi in the wild. However, ONE results in a high density of immunologically naïve kiwi being housed in semi-captive conditions with the potential to result in significant morbidity, and occasionally mortality, from coccidiosis caused by multiple species of *Eimeria*. The aim of this research was to describe any circadian variation in oocyst shedding occurring for the *Eimeria* spp. affecting brown kiwi. Dropping samples were collected from brown kiwi at an ONE site using video surveillance to determine the time of excrement. Oocyst counts were carried out on these droppings and analysed in relation to the time of excrement and the days since the most recent toltrazuril application. The results show that two of the *Eimeria* spp. affecting brown kiwi exhibit circadian variation in oocysts shedding. Oocyst counts for each of the one hour time slots starting at 3am, 4am, 5am and 6am were significantly ($p<0.05$) higher than each of the time slots starting at 8pm, 9pm, 10pm and 11pm. This indicates that peak oocyst shedding occurs between 3am and 7am, with few or no oocysts shed between 8pm and 12pm. The results also suggest high prevalence and abundance of *Eimeria* spp. oocysts in the droppings, with 91% of samples from during peak shedding being positive for *Eimeria* spp., despite recent toltrazuril administration. These findings have several important implications for the conservation of brown kiwi. The reported circadian variation may affect the accuracy of coccidia testing and provides insight into the evolution of this adaptive trait in coccidia. The apparent lack of efficacy of toltrazuril may have management implications and requires further research. The results of this research increase our understanding of the biology of the *Eimeria* spp. affecting brown kiwi. Continuing to improve our understanding of host-parasite interactions is vital to enable effective disease management in order to reduce the detrimental impact of coccidia on

ONE and ensure the ongoing success and sustainability of this important conservation programme.

Preface

This thesis consists of four chapters, including a literature review (Chapter One) and a General Discussion (Chapter Four). The experimental chapters are presented in the style of publishable papers. Chapter Two has been published in *Parasitology Research* (with minor modifications to that presented here), and Chapter Three will be published in the future. These papers have been amended to cite additional information in the appendices, including standard operating procedures and detailed results. In addition the reference lists have been combined into a single reference list and is presented at the end of the thesis. Citations and the reference list follow the APA 6th edition format. As each paper stands alone, there is some repetition between chapters.

Authorship of Chapters

I was the primary author on all work in this thesis, including the published and submitted papers. Dr Kerri Morgan was my chief supervisor and Professor Bill Pomroy and Kate McInnes were co-supervisors.

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Permitting and Animal Ethics Requirements

This research did not involve manipulation of live animals, thus Massey University Animal Ethics approval was not required.

As the collection of samples did not involve disturbance of kiwi a Department of Conservation permit was not required.

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Introduction

The brown kiwi (*Apteryx mantelli*) is a flightless nocturnal avian species endemic to New Zealand and is classified as at risk and in decline (Robertson et al., 2017). Intensive management is carried out by the Department of Conservation (DOC) and community conservation groups to ensure the species has some hope of surviving on mainland New Zealand. The current management and recovery of brown kiwi relies heavily on a captive rearing programme, "Operation Nest Egg" (ONE), and over 200 kiwi are raised in this way each year (Colbourne et al., 2005; Holzapfel et al., 2008). The ONE programme involves removing wild kiwi eggs or young chicks from the nest, hatching any eggs in captivity, raising the chick to a specified age or weight (under either captive or semi-captive conditions) and releasing them back into the wild (Colbourne et al., 2005; Holzapfel et al., 2008). The purpose of ONE is to ensure the main threats to kiwi are mitigated by keeping them in captivity during their most vulnerable life stages (Bassett, 2012; Colbourne et al., 2005). Juveniles are typically released back in to the wild at approximately one kilogram of weight, usually around 4-6 months of age, at which time they are considered big enough to avoid being killed by their main predator, the stoat (*Mustela erminea*) (Bassett, 2012; Colbourne et al., 2005; JA. McLennan, Dew, Miles, Gillingham, & Waiwai, 2004). This captive rearing programme is an important tool for the conservation of kiwi and achieves significant outcomes such as raising the survival to adult-hood from 5% for wild-born kiwi to 65% for ONE kiwi (Colbourne et al., 2005). However this intensive rearing relies on housing immunologically-undeveloped juvenile kiwi at high density with high turnover rates, which may result in pathogen build up (Morgan, Alley, Pomroy, Castro, & Howe, 2012; Morgan et al., 2013; Morgan et al., 2014). One of the most prevalent and limiting diseases influencing the successful rearing of ONE kiwi is coccidiosis (Morgan et al., 2012), and coccidia have been described by Doneley (2006) as the most important gastrointestinal parasite of captive kiwi. Currently little information is available on many aspects of coccidiosis in kiwi (Morgan, 2013) making the management of this

important disease a challenge for kiwi managers. Coccidiosis is estimated to cost the worldwide poultry industry $3 billion annually, and with this economical driver a vast array of research has been conducted on coccidia species affecting domestic chickens (*Gallus gallus*) (Dalloul & Lillehoj, 2006; McDougald, 2003; Peek & Landman, 2011). At present this information is extrapolated to kiwi in order to make management decisions (Morgan, 2013). However, the accuracy of this is unknown and it is vital that our understanding of both the biology of the coccidia species affecting kiwi, as well as the host-parasite interactions, are better understood.

An important interaction that occurs in many other avian coccidia infections is the variable shedding of coccidial oocysts throughout a 24-hour period, known as circadian variation in oocyst shedding. This phenomenon has been described in many different bird species for various species of coccidia (Boughton, 1933; Coelho et al., 2013; Lopez, Figuerola, & Soriguer, 2007; Martinaud, Billaudelle, & Moreau, 2009; Misof, 2004). When present, this variable oocyst shedding pattern limits the accuracy of parasite burden testing via oocyst counts on droppings and if it is not accounted for then the results of such testing are unreliable and may be misleading (Coelho et al., 2013; Filipiak, Mathieu, & Moreau, 2009; Misof, 2004). At present, it is unknown whether circadian variation of oocyst shedding occurs in kiwi, meaning management decisions based on oocyst counts on kiwi droppings may be flawed. The following thesis, and the research therein, seeks to investigate and define any oocyst shedding pattern that may occur in brown kiwi to allow better management of this iconic species. Further to this, the presence and prevalence of different *Eimeria* spp. at the research site is discussed, as is the use of toltrazuril to treat coccidiosis in kiwi.

Chapter One: Literature Review

1.1. Kiwi

Kiwi (*Apteryx* spp.) belong to the order *Casuariiformes* and the family *Apterygidae* and are nocturnal flightless ratites endemic to New Zealand (40.9006° S, 174.8860° E) (Heather & Robertson, 2000; Holzapfel et al., 2008). Brown kiwi (*A. mantelli*) are the most common of the five recognised species of kiwi, however they are still declining in number (Holzapfel et al., 2008; Robertson et al., 2017). Kiwi are slow growing, maturing at 3-5 years of age, and have a life expectancy of 25-50 years (Holzapfel et al., 2008; JA. McLennan et al., 2004). Brown kiwi are generally monogamous and lay 1-2 eggs per clutch, with two clutches per season possible (Holzapfel et al., 2008). Chicks hatch semi-precocial and are never fed by their parents, leaving the nest after about five days and becoming independent by 2-6 weeks of age for some species (Holzapfel et al., 2008). Adult kiwi have a range of 1-100 hectares each, depending on habitat and population density (Castro & Morris, 2011; Holzapfel et al., 2008). Brown kiwi are generally active only at night, spending daylight hours alone in a burrow (Castro & Morris, 2011; JA McLennan, 1988; JA McLennan, Rudge, & Potter, 1987), and typically spend 75% of the night foraging with direct social interaction being rare (Cunningham & Castro, 2011).

1.1.1. Operation Nest Egg

To address the decline of kiwi "Operation Nest Egg" (ONE) was set up in 1995 (Bassett, 2012; Colbourne et al., 2005). In this programme wild kiwi eggs, or recently hatched chicks, are removed from the wild to be hatched and reared in captivity before being released into the wild at approximately one kilogram in weight, at which time they are widely regarded to be predator resistant (Bassett, 2012; Colbourne et al., 2005; JA. McLennan et al., 2004). The ONE programme increases the chance of a kiwi chick surviving to adult-hood to 65% compared with 5% for wild kiwi (Bassett, 2012).

1.2. Coccidia

Coccidia are members of the *Eimeriidae* family (Kingdom Protozoa, Phylum Apicomplexa) (Gussem, 2007). Coccidia are one of the most common intestinal parasites in birds, with *Eimeria* spp. and *Isospora* spp. being the most successful and therefore the most well studied genera (Brown, Ball, & Holman, 2001; Dolnik, 1999c; Lopez et al., 2007; Morgan, 2013). *Isospora* spp. are generally more common in passerines whereas poultry are commonly affected by *Eimeria* spp., however their biology is generally considered to be similar unless proven otherwise (Dolnik, 1999c). For the purposes of this thesis the term 'coccidia' will be used to include all members of the family *Eimeriidae*, as is the common usage.

Coccidia are obligate intracellular parasites that generally complete their complex life cycle in the intestinal tract, although extra-intestinal forms exist (Berto, Flausino, McIntosh, Teixeira-Filho, & Lopes, 2011; Spalding, Carpenter, & Novilla, 2009; Yabsley, 2009). Renal coccidiosis has been reported from several orders of birds however other forms of extra-intestinal coccidia are infrequent in avian species (Morgan et al., 2013; Yabsley, 2009; Yabsley, Gottdenker, & Fischer, 2002). Intestinal species of coccidia are spread via the faecal-oral route with oocysts being produced during the sexual phase of the life cycle and shed in the host's faeces after being released into the intestinal tract (Berto et al., 2011; Martinaud et al., 2009; McDougald, 2003; Yabsley, 2009). Following sporogony (also known as sporulation) outside of the host, oocysts become infective and following ingestion by a susceptible host the oocyst wall is destroyed by grinding in the gizzard, allowing the sporocysts to emerge (Doran & Farr, 1962; Martinaud et al., 2009; Yabsley, 2009). These sporocysts pass to the small intestine where the digestive enzymes facilitate the release of sporozoites from the sporocysts, and, at least for intestinal species of *Eimeria*, allow them to enter the epithelial cells of the wall of the intestine (Conway, 2007; Doran & Farr, 1962). Once in the epithelial cells the sporozoites multiply via merogony (asexual reproduction) to form merozoites which enter new cells and asexually reproduce resulting in cell death and tissue damage (Conway, 2007; McDougald, 2003). Merogony is generally repeated twice, although

some *Eimeria* spp. undergo multiple cycles, with the number of cycles variable and species specific (McDougald, 2003; Yabsley, 2009). These merozoites then undergo gametogony and finally fertilisation to produce new oocysts that are shed in the faeces before sporulating in the environment (Conway, 2007; Dolnik, 1999c; McDougald, 2003; Yabsley, 2009). The exact processes and number of generations of these cycles varies with different coccidia species and will not be covered in detail here.

The time from ingestion of sporulated oocysts to the appearance of the first oocysts in the faeces is known as the prepatent period and can vary considerably, from a few days to weeks, depending on the coccidia species (Joyner & Long, 1974; Long & Joyner, 1984; McDougald, 2003). Sporulation requires particular environmental conditions, including the presence of oxygen, adequate humidity and an ideal temperature, and takes from 10 hours to seven days depending on the species (Dolnik, 1999c; Dolnik, Metzger, & Loonen, 2011; Martinaud et al., 2009; McDougald, 2003; Yabsley, 2009). Unsporulated oocysts are particularly vulnerable to damage due to desiccation or sun exposure but once sporulated, oocysts are resistant to a range of environmental conditions and may survive up to 18 months, although most reports indicate shorter survival times than this (Berto et al., 2011; Delaplane, 1935; Dolnik, 1999c; Martinaud et al., 2009).

Coccidia are generally homoxenous (have a single host) or have narrow host specificity and are unable to complete their lifecycle in a non-host species (Berto et al., 2011; Dolnik, 1999c; Marquardt, 1981; Morgan, 2013; Schoener, Alley, Howe, & Castro, 2013; Yabsley, 2009). Historically, host specificity was not recognised, with over 100 species of passerines having been reported as hosts of *I. lacazei* (Berto, Luz, Ferreira, Flausino, & Lopes, 2010). However, based on descriptions and the improbability that one species could parasitise so many hosts, Levine (1982) considered it more likely that each host was infected by its own species of *Isospora* and proposed the concept of host specificity (N. D. Levine, 1982). Since these early findings there has been some disagreement on the degree of host specificity across the literature. Some authors have

proposed intra-familial specificity where cross transmission is limited to hosts from different genera within the same family (Berto et al., 2010; Duszynski & Wilber, 1997), and others have proposed intra-genus specificity (Joyner & Long, 1974; Tung et al., 2007). Marquardt (1981) hypothesised that coccidia could only cross-infect closely related host species, with cross-infection between hosts in other genera rare, and cross-infection between hosts in other taxonomic families almost never occurring. However, the author accepts there are exceptions to this (Marquardt, 1981). Tung et al. (2007) showed that a species of *Isospora* did not infect hosts from other genera but did infect two hosts from the same genus. In contrast, Berto et al. (2010) describes the same species of *Isospora* affecting three passerine host species from different genera but within the same family. Finally, Norton (1967) reports infections in turkeys (*Meleagris* spp.) following inoculation with *E. colchici* from English pheasants (*Phasianus colchicus)* (Norton, 1967), showing this *Eimeria* spp. infects hosts from different families and indicating there are at least some exceptions to the general rule. Thus further research is needed in this area to be able draw firm conclusions as *Eimeria* spp. host specificity varies between species.

1.2.1. Coccidiosis

Coccidiosis is the term used to refer to infection with coccidia and may be clinical or subclinical, depending on the infection intensity and subsequent expression of clinical signs (Yabsley, 2009). During their lifecycle, coccidia cause cellular damage which may impair intestinal function and nutrient uptake, (Gussem, 2007; Long, 1987; McDougald, 2003; Yabsley, 2009), as well as potentially impairing other infected organs such as the kidneys or liver (Morgan et al., 2013; Yabsley, 2009; Yabsley et al., 2002). Infection with coccidia may also predispose the affected host to secondary disease such as infection by *Clostridium* spp. (Gussem, 2007; Long, 1987; McDougald, 2003). Subclinical coccidiosis may result in impaired performance, reduced feed conversion efficiency and reduced growth in affected animals (Gussem, 2007; Long, 1987; McDougald, 2003). Clinical coccidiosis may result in a range of clinical signs including weight loss, reduced appetite, diarrhoea, dysentery, dehydration and weakness, and may lead to

death (Berto et al., 2011; Brawner III & Hill, 1999; Dolnik, 1999c; Gussem, 2007; Long, 1973, 1987; McDougald, 2003; Peek & Landman, 2011; Thompson & Wright, 1978; Yabsley, 2009). The severity of coccidiosis is determined by a number of factors including the pathogenicity of the coccidia species, the infective dose, and various host factors, for example age and concurrent disease (Long, 1973, 1987; McDougald, 2003; Yabsley, 2009). The severity of disease is generally thought to be dose dependent with more oocysts being ingested leading to more damage and therefore more severe disease (Long, 1973; Yabsley, 2009). However, Filipiak et al. (2009) found that the number of oocysts shed in the faeces was independent of the infective dose and went on to speculate that the number of oocysts that successfully establish and multiply is consistent despite the infective dose, possibly limited by the number of available intestinal epithelial cells, although evidence to support this theory appears lacking. High stocking rates and stress, such as that experienced during handling, also lead to more common and more severe disease (Peek & Landman, 2011; Yabsley, 2009) and younger individuals are generally assumed to be more susceptible to coccidiosis which is possibly due to acquired immunity in older individuals (Dolnik, 1999c; McDougald, 2003).

Diagnosis of coccidiosis relies on either histological examination of infected tissues collected at post-mortem examination or counting the oocysts present in faeces (Yabsley, 2009). In the poultry industry, post-mortem examinations may be routinely carried out, however in wildlife management this is rare and sporadic, meaning the non-invasive technique of faecal oocyst counts is more appropriate and commonly employed.

1.2.2. *Eimeria* spp.

Using morphological techniques, *Eimeria* spp. are differentiated from other coccidia genera by the presence of four sporocysts each containing two sporozoites, compared with the oocysts of *Isospora* spp. which contain two sporocysts with four sporozoites in each (Soulsby, 1982; Yabsley, 2009). In general, the reproductive potential (the number of oocysts produced per oocyst ingested) is high for *Eimeria* spp. although this

potential varies considerably between species (Brackett & Bliznick, 1952; Yabsley, 2009). In general, asexual reproduction of *Eimeria* spp. does not continue indefinitely meaning that without reinfection an infection is self-limiting and will run its course (McDougald, 2003).

Host immunity to *Eimeria* spp. develops in response to repeated exposure following several cycles of infection and recovery and is not necessarily age related (Long, 1987; Shirley & Lillehoj, 2012; Yun, Lillehoj, & Lillehoj, 2000). Exposure to one species of *Eimeria* may not infer cross-immunity to other *Eimeria* spp. (Joyner & Long, 1974; Long & Joyner, 1984; Yabsley, 2009) meaning an individual is unlikely to have immunity to a species of *Eimeria* to which they have not previously been exposed. Although immunity does not guarantee the absence of infection, it can help to limit the effects of cocccidiosis via reduced clinical signs, reduced pathological effects, and reduced parasite replication within the host (Dolnik, 1999c; Shirley & Lillehoj, 2012; Yun et al., 2000).

Significant variation between *Eimeria* spp. in both the prepatent period and the oocyst sporulation time means the life cycle lengths vary considerably depending on the species. For example, in species of *Eimeria* affecting poultry the prepatent period varies from three and a half days (for *E. praecox)* to six days (for *E. necatrix)* and oocycst sporulation times vary from 12 to 30 hours for the various *Eimeria* spp. affecting poultry (*Gallus gallus*) (McDougald, 2003).

1.3. Coccidia in kiwi

Coccidia are a relatively common parasite of kiwi (Doneley, 2006; Jakob-Hoff, Buchan, & Boyland, 1999; Morgan, 2013; Morgan et al., 2012; Morgan, Pomroy, Howe, Alley, & Castro, 2017) and are currently seen as the most important parasite affecting kiwi in captivity (Doneley, 2006; Morgan, 2008). Coccidia are a naturally occurring parasite of wild kiwi (Bassett, 2012; Jakob-Hoff et al., 1999) and are found in wild populations that have never come in contact with ONE raised kiwi (Jakob-Hoff et al., 1999). In wild, other-wise healthy individuals, a low level of infection with coccidia does not

necessarily cause clinical illness (Jakob-Hoff et al., 1999; Morgan et al., 2014). Morgan et al. (2017) describes four species of *Eimeria* affecting kiwi and coccidia have been found in all five species of kiwi (Morgan et al., 2017). However, host specificity of the *Eimeria* spp. affecting kiwi is not understood and further information is required to determine the distribution of *Eimeria* spp. between different kiwi species and populations (Morgan et al., 2014; Morgan et al., 2017). Intestinal, hepatic, renal, splenic and pulmonary forms of coccidial infection have been diagnosed in kiwi via post-mortem examination with intestinal being the most common, followed by renal then hepatic and pulmonary and splenic being uncommon (Morgan et al., 2012; Morgan et al., 2013; Morgan et al., 2014). Under natural conditions it is likely that juvenile kiwi would be exposed to low levels of coccidia and slowly develop immunity as a response to repeated exposure (Bassett, 2012; Morgan, 2013; Morgan et al., 2014). Coccidiosis may be associated with significant morbidity and mortality in kiwi. A retrospective survey of 372 post-mortem examinations on kiwi found that 13% of birds were positive for coccidia and that coccidia was the primary cause of death in 26% of positive cases (Morgan et al., 2013; Morgan et al., 2014). Habitat is a factor in the prevalence of coccidia in avian species. It has been observed that ground feeding and forest dwelling avian species generally have a higher prevalence (McQuistion, 2000). Consequently, kiwi may be expected to have a higher prevalence than previously studied avian species. Jakob-Hoff et al. (1999) found that 50% of captive reared kiwi and 29% of wild born kiwi were positive for coccidia based on oocyst counts on droppings during a survey. Morgan et al. (2014) found 24% of 405 dropping samples from various captive and wild kiwi populations to be positive for oocysts. However, the author suggests that analysing a sample from a single point in time is likely to under-estimate the true prevalence of coccidia due to possible variation in oocyst shedding throughout the day, citing unpublished data that showed an individual kiwi sampled over 24 hours exhibited oocysts counts varying from 0 to 10000 oocysts per gram (Morgan et al., 2014). This possible existence of circadian variation in oocyst shedding

of coccidia in kiwi has not been previously investigated and is the focus of this present research.

1.3.1. Coccidia and ONE

Under natural wild conditions, host and coccidia can likely coexist with only mild or subclinical disease evident, with severe disease generally only seen in cases of overcrowding, stress and/or immunosuppression (McQuistion, 2000; Morgan et al., 2014; Shirley & Lillehoj, 2012; Yabsley, 2009). However, the process of ONE results in several known risk factors for coccidia occurring in conjunction. Intensive rearing is a known risk factor for coccidiosis (McDougald, 2003; Peek & Landman, 2011; Schoener et al., 2013). Wild kiwi generally have a range of 1-100 hectares (Castro & Morris, 2011; Holzapfel et al., 2008). In contrast, ONE kiwi are only required to have a minimum area of $15m^2$ per chick once in outside runs (Bassett, 2012). This increased stocking rate of kiwi within ONE facilities is likely to markedly increase environmental contamination with coccidial oocysts, resulting in an increased chance of higher oocyst ingestion rates (McQuistion, 2000; Morgan, 2013; Morgan et al., 2014). Young age is also a risk factor for coccidiosis in kiwi (Jakob-Hoff et al., 1999; Morgan et al., 2013; Morgan et al., 2014). Thus the presence of juvenile kiwi, which are likely to be immunologically naïve to coccidia, may result in more frequent clinical disease at ONE facilities (Jakob-Hoff et al., 1999; Morgan, 2013; Morgan et al., 2013; Morgan et al., 2014). Coccidia are most prevalent in juvenile kiwi (under 18 months of age) (Morgan et al., 2013; Morgan et al., 2014) and extra-intestinal coccidiosis affects juvenile kiwi more often than in mature birds (Morgan et al., 2013). Thus, as ONE often results in both an increased density of juvenile kiwi and the potential for increased stress, for example from handling, it is probable that ONE birds are more likely to experience clinical disease than their wild counterparts (Morgan et al., 2013; Morgan et al., 2014).

Under favourable conditions, coccidia oocysts can survive many months in the environment (Bassett, 2012; Delaplane, 1935; Yabsley, 2009) and with new groups of naïve juveniles regularly passing through size-limited ONE facilities there is potential for the environmental burden of coccidia to exceed those naturally occurring in the

wild. Ultimately, ONE facilities appear to combine several known risk factors for coccidia and it is likely that the coccidiosis affecting ONE kiwi is due to a dose dependant response to increased environmental contamination. This is due to the high stocking rate of immunologically naïve kiwi cycling through ONE facilities on a yearly basis (Bassett, 2012; Jakob-Hoff et al., 1999; Morgan et al., 2012; Morgan et al., 2013; Morgan et al., 2014).

1.3.2. Species of coccidia affecting kiwi

Identification and description of causative agents is vital in understanding and managing coccidiosis in kiwi. Thompson & Wright (1978) describe the morphology of four types of coccidial oocysts found in kiwi dropping samples. These authors reported that further identification was not possible as attempts to sporulate them were unsuccessful (Thompson & Wright, 1978). However, Morgan et al. (2017) did manage to successfully sporulate oocysts from brown kiwi droppings allowing a more comprehensive description of four species of *Eimeria* found in brown kiwi; *E. paraurii, E. apteryxii, E. mantellii* and *E. kiwii.* Although yet to be conclusively determined, Morgan et al. (2017) suggests that *E. paraurii* and *E. kiwii* are responsible for intestinal forms of coccidiosis while *E. apteryxii* may cause a renal form. Further research is required to confirm the tissues that each species infects, as well as to determine relative pathogenicity (Morgan et al., 2017). Although intestinal and renal forms of coccidiosis are common in avian hosts, other extra intestinal forms are rare (Morgan et al., 2013; Yabsley, 2009). Naturally occurring hepatic coccidiosis is rarely reported in avian hosts (Morgan et al., 2013; Yabsley, 2009). Splenic and pulmonary coccidiosis have been reported in cranes (*Grus* spp.) as part of disseminated visceral coccidiosis (Novilla & Carpenter, 2004). Morgan et. al. (2013) found that extra intestinal coccidial infection was common in kiwi. Interestingly, they reported hepatic, splenic and pulmonary forms as well as the more common intestinal and renal forms (Alley, 2003, 2004; Morgan, 2013; Morgan et al., 2013). This suggests kiwi are unusual in the existence of several extra-intestinal forms of *Eimeria* spp. infection.

Morgan et al. (2017) found *E. apteryxii* and *E. kiwii* were the most common species excreted by kiwi, occurring in all six samples examined from two research sites. The less commonly found *E. paraurii* only occurred in two kiwi from one research site and *E. mantellii* was only found in one bird from the other research site (Morgan et al., 2017). As the oocysts used to describe these four species were collected from only six kiwi at two geographical locations, it is likely that additional species of coccidia affecting kiwi may exist (Morgan et al., 2017). Further to this, Morgan et al. (2017) only used samples from brown kiwi and, as coccidia have been identified histologically in all five species of kiwi (Morgan et al., 2014), it is also likely there are other *Eimeria* spp. affecting these other kiwi species (Morgan et al., 2017). The host specificity of the four described *Eimeria* spp. from brown kiwi is not yet understood (Morgan et al., 2017), and it is unknown if they may infect other species of kiwi. The five separate species of kiwi are all from the same taxonomic family and genera (Burbidge, Colbourne, Robertson, & Baker, 2003; Holzapfel et al., 2008), meaning it is possible that they may be susceptible to the same species of coccidia. However further research is required to investigate this. It is also important to note that individual kiwi may carry more than one species of *Eimeria* concurrently (Morgan et al., 2017; Thompson & Wright, 1978).

Importantly, Morgan et al. (2017) describes how to sporulate oocysts from *Eimeria* spp. affecting kiwi, a task that has proven difficult in comparison to other species, allowing future use of this technique in any subsequent research . Interestingly, Morgan et al. (2017) reports that oocysts sporulated within 7-10 days, which is significantly longer than is reported in other avian host species (Dolnik, 1999c; McDougald, 2003). However, as the author only observed the oocysts between days seven and 10, and not at regular intervals up to this time, sporulation may have occurred at any time within this period and further research is required to determine a precise sporulation time for these species. Sporulation takes 12 to 30 hours for the *Eimeria* spp. affecting poultry (McDougald, 2003), suggesting that sporulation times for *Eimeria* spp. affecting brown kiwi are likely to be considerably less than 10 days. Morgan et al. (2017) also noted that despite extensive examination of oocysts from kiwi, no spontaneously sporulated

oocysts were observed despite significant periods of time at room temperature. This is difficult to explain and the author suggests it may be related to the forest dwelling nature of kiwi whereby the oocysts may require cool, damp conditions to sporulate (Morgan et al., 2017). This is supported by McQuistion (2000) who stated that the damp and humid conditions found within forests are conducive to oocyst development. The sporulation of oocysts for *Eimeria* spp. affecting brown kiwi is an area requiring further investigation.

1.3.3. Implications for wild kiwi populations

Failure to successfully control coccidia in ONE facilities will not only result in morbidity and mortality of ONE juveniles but may also have flow on effects to wild kiwi populations upon release of infected individuals (Morgan et al., 2012; Morgan et al., 2013; Morgan et al., 2014). The release of coccidia-infected ONE kiwi into the wild is likely to pose little threat to the resident wild kiwi, as without the inciting risk factors occurring in captivity the presence of coccidia alone is unlikely to progress to clinical disease in other-wise healthy kiwi (Jakob-Hoff et al., 1999; Morgan et al., 2013; Morgan et al., 2014). However, an area of uncertainty is the possibility of introducing a locally novel species of coccidia into a naïve population of wild kiwi who may lack immunological protection to that particular coccidia species, possibly resulting in a disease outbreak (Morgan et al., 2013; Morgan et al., 2014). If a wild kiwi population has not previously encountered a particular *Eimeria* spp. it may lack immunity to it and subsequent exposure to that *Eimeria* spp., via a released ONE kiwi, is a potential threat (Morgan et al., 2013; Morgan et al., 2014). This principle also applies in reverse whereby released ONE kiwi may be exposed to a novel *Eimeria* spp. present in the wild population and reduced post-release survival may result. However until the composition of the various species of *Eimeria* affecting kiwi is understood across kiwi populations this threat cannot be quantified. Morgan et al. (2017) found differences in *Eimeria* spp. presence between the two ONE study sites during her research and these differences suggest that different kiwi populations may have different *Eimeria* spp. present. However, Morgan et al. (2017) received samples from only six kiwi across two

captive facilities and as such it is difficult to draw conclusions on the *Eimeria* spp. composition of these two populations.

ONE is currently carried out on all five species of kiwi, with many ONE facilities rearing more than one species at their facilities and, as all species of kiwi have had reported cases of coccidia (Morgan et al., 2013; Morgan et al., 2014), there may be a significant risk of the unnatural transfer of coccidia between kiwi species should they be susceptible (Morgan, 2013; Morgan et al., 2013). However further research is needed in this area to draw firm conclusions as *Eimeria* spp. host specificity varies between species (Berto et al., 2011; Dolnik, 1999c; Marquardt, 1981; Tung et al., 2007) and is currently unknown for the *Eimeria* spp. affecting kiwi.

1.3.4. Current management of coccidiosis in kiwi

Establishing baseline data on infectious agents is a vital requirement for recovery programmes (Coelho et al., 2013) and many ONE facilities carry out various routine testing regimes to facilitate this. Having an accurate and consistent estimate of the intensity of the coccidia burden within a population is vital to determine trends and evaluate the threat from coccidia. Morgan (2008) suggests pooling samples from throughout a 24-hour period due to the possibility of diurnal variance in oocyst shedding. There are currently no best practice guidelines for sample collection and due to the variable management techniques and resources of different ONE facilities, there is wide variation in the design of surveillance programmes carried out across the different facilities (*personal observation*). Within ONE kiwi rearing facilities, intervention is often routinely required to prevent significant mortality from coccidiosis.

1.4. Toltrazuril

1.4.1. Mechanism of action

Toltrazuril, a symmetrical triazinone, has been shown to be a highly efficacious anticoccidial compound for treating coccidiosis and has a long record of use in poultry medicine (Chapman, 1987; Claeskens, Verdonck, Heesen, Froyman, & Torres, 2007).

Unlike many other anticoccidial drugs that only act on free stages of coccidia, toltrazuril is efficacious against all intracellular stages (merogony and gametogony) and it induces cidal changes in the organelles of the coccidia at various levels, however already formed oocysts do not appear to be destroyed (Greif, 2000; Harder & Haberkorn, 1989; Mehlhorn, Ortmann-Falkenstein, & Haberkorn, 1984; Peek & Landman, 2011). The exact mode of action for toltrazuril is unknown however it is likely to effect the mitochondrial action of the coccidia (Harder & Haberkorn, 1989; Mehlhorn et al., 1984). Toltrazuril is metabolised into toltrazuril sulfone or ponazuril, both of which are able to pass through the host cell membrane and cytoplasm, where they cause swelling of the mitochondria and enlargement of the endoplasmic reticulum of the parasite, thus affecting the intracellular developmental stages of the coccidia (Mehlhorn et al., 1984). Further to this, Harder et. al. (1989) proposed that toltrazuril affected the mitochondrial respiratory chain and two enzymes involved in pyrimidine synthesis by the parasite. It appears that, despite its widespread use, the mechanism of action for toltrazuril is not well understood or explained in detail in the literature.

1.4.2. Toltrazuril use in avian species

Previous studies of toltrazuril against other species of coccidia suggest that it results in either complete elimination or highly significant reductions in the number of oocysts being shed within several days of treatment (Chapman, 1987; Ramadan, El-Sooud, & El-Bahy, 1997; Sokół, Gesek, Raś-Noryńska, & Michalczyk, 2014; Van Reeth & Vercruysse, 1993). Research in pigeons (*Columba* spp.) that investigated the efficacy of toltrazuril against *Eimeria* infections showed a decrease of 97-99% in oocyst counts within three to four days of treatment (Van Reeth & Vercruysse, 1993). A study of toltrazuril use against *Eimeria* spp. in poultry showed an 88-100% reduction in oocyst counts following treatment, however timeframes were not provided for the treatment to testing interval (Vertommen, Peek, & Van der Laan, 1990). Another study on various *Eimeria* spp. affecting poultry showed a large reduction in oocyst output two days after toltrazuril administration (Ramadan et al., 1997). Oocyst counts on faeces of Japanese quails (*Cotumix cotumix japonica)* infected with *Eimeria* spp. began decreasing on day

two following toltrazuril administration and counts reached zero by three to five days post treatment (Sokół et al., 2014). Toltrazuril has the advantage of being effective after a single treatment and it appears to take about 24-48 hours for the full effect to be evident in chickens (Mehlhorn et al., 1984; Peek & Landman, 2011; Syed-Hussain, 2014).

Toltrazuril has been shown to have persistent activity in some avian host species. Van Reeth & Vercruysse (1993) found that treatment of *Eimeria* spp. in pigeons with toltrazuril up to 14 days before experimental infection resulted in a 97% decrease in average oocyst counts compared to unmedicated controls (Van Reeth & Vercruysse, 1993). This concurs with earlier work in poultry showing that toltrazuril inhibits the development of *Eimeria* spp. for up to seven days (Vertommen et al., 1990). These results suggest that toltrazuril may provide long lasting activity in avian species, impairing the development of coccidia for 7-14 days (Van Reeth & Vercruysse, 1993; Vertommen et al., 1990).

The use of toltrazuril does not impair the development of natural immunity to coccidia (Chapman, 1999; Greif, 2000; Peek & Landman, 2011). However, repeated exposure to anticoccidials may lead to the development of drug resistance (Chapman, 1997; Peek & Landman, 2011; Stephan, Rommel, Daugschies, & Haberkorn, 1997; Vertommen et al., 1990). The development of toltrazuril resistance has been reported in two previous studies, both for the *Eimeria* spp. affecting poultry (Stephan et al., 1997; Vertommen et al., 1990). Vertommen et al. (1990) found that administration of toltrazuril prevented coccidiosis for several weeks in poultry broilers, however after five consecutive rearing periods coccidiosis emerged. The authors then investigated the efficacy of toltrazuril against the *Eimeria* spp. from this fifth flock, referring to them as a strain, as well as two other strains of *Eimeria* from farms were coccidiosis occurred despite toltrazuril administration. This was done via artificial infection with three different levels of each strain followed by toltrazuril application and subsequent faecal oocyst counts. They found that one strain was resistant to toltrazuril, one strain was tolerant and one strain

was fully susceptible to toltrazuril. These results suggest that resistance to toltrazuril will develop due to application over an extended period of time (Vertommen et al., 1990). However, coccidiosis occurred on the farm where the susceptible strain was collected, despite toltrazuril application, suggesting that resistance is not always the cause of treatment failure (Vertommen et al., 1990). Stephan et al. (1997) tested the efficacy of toltrazuril against ten strains of mixed *Eimeria spp*. isolated from different poultry farms. Again, this was achieved via artificial infection and subsequent toltrazuril administration. One of these strains was found to be resistant to toltrazuril, while nine of the ten were resistant to at least one other anticoccidial.

Finally, it is suggested that toltrazurils efficacy is related to the infective dose of oocysts (Vertommen et al., 1990). Vertommen et al. (1990) found subsequent oocyst counts to be related to the infective dose despite all birds being treated with toltrazuril, and concluded that toltrazuril performed better under low coccidian challenges.

1.4.3. Toltrazuril use in kiwi

Toltrazuril is routinely used to treat coccidiosis in kiwi at an oral dose rate of 25mg/kg (*Baycox^{TM} coccidiocide for piglets, 50mg/ml, Bayer, Leverkusen, Germany*) (Doneley, 2006; Morgan, 2008). It is commonly used by ONE facilities to control coccidia. However, historical data or case reports on the efficacy of toltrazuril use in kiwi are non-existent. The frequency of toltrazuril dosing varies considerably between captive kiwi facilities and it is important to undertake prudent use of this drug, as overuse risks the development of resistance (Morgan, 2013).

1.4.4. Toltrazuril use for extra-intestinal coccidiosis

The use of toltrazuril for treating extra-intestinal forms of coccidiosis is not reported in the literature. Thus it is currently unknown if toltrazuril is efficacious against some of the extra-intestinal forms of coccidiosis described in kiwi. However, extra-intestinal coccidiosis has been reported in several species of crane (*Grus* spp.), described as disseminated visceral coccidiosis (DVC) (Novilla & Carpenter, 2004; Spalding et al.). There are reports of the use of other anticoccidials to treat DVC in cranes (Carpenter,

Novilla, & Hatfield, 2005; Spalding et al.), with some being shown to lack efficacy against DVC (Carpenter et al., 2005). The author states that unless an orally administered drug is absorbed in sufficient quantities to produce systemic activity, control of extra-intestinal coccidia species may be difficult (Carpenter et al., 2005).

1.5. Diurnal periodicity of oocyst shedding in birds

Coccidia prevalence and burden intensity is known to vary annually, seasonally, and geographically for avian hosts (Berto et al., 2011; Dolnik, 1999c; Misof, 2004). In addition, circadian variation of coccidial oocyst shedding has been observed in a number of avian host species (Table 1) (Berto et al., 2011; Dolnik, 1999b; Martinaud et al., 2009; Misof, 2004). The seasonal and annual fluctuations are likely to be 'real' whereas diurnal fluctuations are likely to represent fluctuations in parasite detectability, rather than their true prevalence or abundance (Misof, 2004). This circadian variation in oocyst shedding has now been documented in a range of avian host species in several different taxa (Boughton, 1933, 1937c; Brawner III & Hill, 1999; Brown et al., 2001; Coelho et al., 2013; Dolnik, 1999a, 1999b; Dolnik et al., 2011; Filipiak et al., 2009; Hudman, Ketterson, & Nolan Jr, 2000; P. P. Levine, 1942; Lindström et al., 2009; Lopez et al., 2007; Martinaud et al., 2009; Misof, 2004; Morin-Adeline et al., 2011; Villanúa, Pérez-Rodríguez, Gortázar, Höfle, & Viñuela, 2006). In general, oocyst output has shown a large variation at different times of the day with a general pattern of higher prevalence and larger numbers of oocysts in the late afternoon compared with the morning and night (Brawner III & Hill, 1999; Coelho et al., 2013; Dolnik, 1999c; Dolnik et al., 2011; Filipiak et al., 2009; Lindström et al., 2009; Lopez et al., 2007; Martinaud et al., 2009; Villanúa et al., 2006). The majority of the coccidia species for which this circadian pattern has been described are *Isospora* spp. with only Boughton (1937c)**,** Levine (1942) and Villanua et al. (2006) demonstrating a diurnal shedding pattern in *Eimeria* spp. affecting avian hosts. The circadian variation of coccidia oocyst shedding is a particularly well described phenomenon in passerines (order *Passeriformes*) (Dolnik et al., 2011) with comprehensive data for other orders being more limited. Boughton (1933) was the first to describe the circadian variation in

oocyst shedding and, although these early observations did not gain much attention at the time (Dolnik, 1999b), the gradual understanding of host-parasite interactions has led to a renewed interest in such relationships and the potential biological mechanisms behind them. As well as this, the need to be able to accurately assess the coccidia burden of a managed population of bird species, which is not possible without an understanding of the oocyst shedding cycle (Coelho et al., 2013; Misof, 2004), has garnered further interest in this topic. As a result several studies have now been carried out on the circadian variation of oocyst shedding across various host species and are summarised in Table 1 and discussed below.

1.5.1. Findings of previous research

The circadian cycle of shedding of coccidia oocysts was first reported in English sparrows (*Passer domesticus*) by Boughton (1933), who described the periodic release of *Isospora* spp. oocysts with peak shedding occurring between 3pm and 8pm. Boughton went on to publish several papers on this phenomenon (Boughton, 1933, 1937a, 1937b, 1937c; Boughton, Atchley, & Eskridge, 1935), culminating in the hypothesis of a 'circaduodian rhythm' whereby coccidia follow a 48 hour pattern, with two separate populations generally present giving the impression of a 24 hour pattern (Boughton, 1988). Although this hypothesis is no longer referred to in the literature, this early work was vital in raising awareness of circadian variation in oocyst output. Further to this, as no statistical analysis was evident for any of Boughton's work, robust specific conclusions cannot be drawn, however general trends remained evident. Since Boughtons earlier work this phenomenon has been described in various coccidian species across a range of avian hosts. When investigating the circadian variation of oocyst shedding in droppings for other avian host species some authors have investigated presence or absence (prevalence) only (Brown et al., 2001; Dolnik et al., 2011; Hudman et al., 2000; Lindström et al., 2009), while others have investigated both the prevalence and abundance of oocysts (Brawner III & Hill, 1999; Coelho et al., 2013; Dolnik, 1999a, 1999b; Filipiak et al., 2009; P. P. Levine, 1942; Lopez et al., 2007; Misof,

2004; Morin-Adeline et al., 2011; Villanúa et al., 2006), showing that both follow the same general pattern.

Table 1
A summary of the results of previous research on the circadian variation in oocyst shedding for several species of coccidia affecting various avian host species.

Host Species	**Number of hosts (total samples)**	**Hosts Wild, Wild Captured or Captive**	**Coccidia Species**	**Time slots for sampling (*indicates time of peak shedding)**	**Presence only or Abundance**	**Reference**
English sparrow (*Passer domesticus*)	10 (*not stated*)	Wild Captured	*Isospora* spp.	9am-3pm, 3-8pm*	Abundance	Boughton, 1933
English sparrow (*Passer domesticus*)	30 (*not stated*)	Wild Captured	*Isospora* spp.	9am-3pm, 3pm-9pm*, 9pm-3am, 3am-9am	Abundance	Boughton, 1937b
Pigeons (*Columba* spp.)	5 (*not stated*)	Wild Captured	*E. labbeana*	9am-3pm*, 3pm-9pm, 9pm-9am	Abundance	Boughton,1937c
Chickens (*Gallus gallus*) *(Note; inoculated infections)*	50 (*not stated*)	Captive	*E. praecox, E. mitis, E. maxima, E. hagani, E. necatrix*	9am-3pm, 3pm-9pm****, 9pm-9am*	Abundance	Levine, 1942
House finches (*Carpodacus mexicanus*)	26 (26)	Wild Captured	*Isospora* spp.	Before 10am, After 3pm*	Presence	Brawner & Hill, 1999
	8 (*32*)	Wild Captured	*Isospora* spp.	8am, 12pm, 4pm*, 7pm*	Abundance	
Common starlings (*Sturnus vulgaris*)	100 (100)	Wild Captured	*I. dilatata*	8am, 9am, 10am, 11am, 12pm, 1pm, 2pm, 3pm, 4pm*,5pm*,7pm*,8pm*, 9pm	Abundance	Dolnik, 1999a
Starlings (*Sturnus vulgaris*), Scarlet grosbeaks (*Carpodacus erythrinus*), Garden	362 (362)	Wild Captured	*Isospora* spp.	7am, 8am, 9am, 10am, 11am, 12pm, 1pm, 2pm, 3pm, 4pm*, 5pm*, 6pm*, 7pm*, 8pm*, 9pm, 10pm, 11pm	Abundance	Dolnik, 1999b

warblers (*Sylvia borin),* Reed warblers (*Acrocephalus scirpaceus*), Willow warblers (*Phylloscopus trochilus*), Chaffinches (*fringilla coelebs*)	14 (*not stated*)	Wild Captured		1am, 2am, 3am, 4am, 5am, 6am, 7am, 8am, 9am, 10am, 11am, 12pm, 1pm*, 2pm*, 3pm*, 4pm*, 5pm*, 6pm*, 7pm*, 8pm*, 9pm*, 10pm, 11pm, 12am		
Dark eyed juncos (*Junco hyemalis*)	67 (108)	Wild Captured	*Isospora* spp.	3pm-5am*, 5am-3pm	Presence	Hudman et al., 2000
Green finch (*carduelis chloris*)	404 (404)	Wild Captured	*Isospora* spp.	7-8am, 8-9am ,9-10am, 10-11am, 11-12pm, 12-1pm,1-2pm, 2-3pm*, 3-4pm*, 4-5pm*, 5-6pm,6-7pm	Presence	Brown et al., 2001
Eurasian blackbirds (*Turdus merula*)	57 (114)	Wild	*Isospora* spp.	6am-12pm, 12pm-6pm*	Abundance	Misof, 2004
Garden warbler (*Sylvia borin*), European serin (*Serinus serinus*)	406 (406)	Wild Captured	*Isospora* spp.	Times standardised to hours after sunrise ; Peak from ½ to 9/10 of daylight period	Abundance	Lopez et al., 2007
Eurasian blackbirds (*Turdus merula*)	10 (80)	Wild Captured	*I. turdi*	7am-9am, 9am-11am, 11am-1pm, 1pm-3pm, 3pm-5pm, 5pm-7pm*, 7pm-9pm, 9pm-7am	Abundance	Filipiak et al., 2009
Ground finches (*Geospiza fuliginosa*)	219 (219)	Wild Captured	*I. fragmenta, I. temeraria, I. exigua*	Morning (before 12pm) Afternoon (after 12pm)*	Presence	Lindstrom et al., 2009
Regent honeyeater (*Xanthomyza phyrigia*)	53 (174)	Captive	*I. lesouefi*	8-11am 2.30-5.30pm*	Abundance	Morin-Adeline et al., 2011
Red-legged partridges (*Alectoris rufa*)	6 (167)	Captive	*Eimeria* spp.	8am, 12pm, 4pm*, 7.30pm*	Abundance	Villanua et al., 2006
Snow bunting (*Plectrophenax nivalis*)	100 (250)	Wild	*I. plectrophenaxia*	2am,4am,6am,8am,10am,12pm, 2pm,4pm*,6pm 8pm,10pm,12am	Presence	Dolnik et al., 2011
Green winged saltator (*Saltator similis*)	164 (220)	Wild Captured	*I. similisi*	9am-12pm, 3-5pm*	Abundance	Coelho et al., 2013

1.5.1.1. Circadian variation in oocyst shedding in Eimeria spp.

One of the few studies to investigate the phenomenon of circadian variation in oocyst shedding in *Eimeria* spp. was Boughton (1937). The author sampled five caged pigeons (*Columba* spp.) across three time slots and found peak oocyst shedding occurred between 9am and 3pm. When comparing these results to his earlier work on *Isospora* spp. in sparrows, the author concluded that *Eimeria* spp. peaked earlier than *Isospora* spp. In contrast, Levine (1942), who also investigated *Eimeria* spp. but in chickens (*gallus gallus*), found peak shedding to occur between 3pm and 9pm for four of the five species investigated. This later work suggests *Eimeria* spp. follow a similar pattern to *Isospora* spp. in those host species studied. Boughton (1988) reviews his previous work on *Eimeria* spp. in light of Levine's (1942) findings and suggests that the pigeons in his original study were probably shedding earlier in the day due to the season, rather than a generic difference between *Eimeria* and *Isospora* spp.

Levine's (1942) research represents the first report of variation between different coccidial species within the same host, the chicken. By dosing groups of birds with different *Eimeria* spp. the author was able to compare five *Eimeria* spp., finding that all showed a clear diurnal pattern of oocyst shedding, with four of the five species demonstrating peak shedding between 3pm and 9pm and the other peaking between 9pm and 9am. Interestingly, the author excluded *E. tenella*, a commonly occurring species in poultry, due to its formation of caseous plugs in the caecal lumen, which he proposed would affect the discharge of oocysts from that organ (P. P. Levine, 1942). Rectifying this omission, Boughton (1988) sought to investigate the circadian periodicity of *E. tenella.* However the author did this by post-mortem examination of the caeca rather than the more conventional method of faecal oocyst counts. Boughton (1988) concluded that *E. tenella* underwent circadian periodicity and that the timing of this cycle depended on both the time of inoculation and some other, unknown, factor. This research had several limitations to which Boughton himself alludes, including the attempted analysis of concurrent drug treatment. Nevertheless

these early observations were vital in raising awareness of the variation in oocyst output over time.

More recently, Villanua et al. (2006) investigated the circadian variation of oocyst shedding for *Eimeria* spp. affecting red-legged partridges (*Alectoris rufa*). The findings showed that the time of sample collection had a significant effect on oocyst counts with a trend of increasing oocyst counts throughout the day, with maximum output occurring in the late afternoon (Villanúa et al., 2006). Interestingly, Villanua et al. (2006) reports variation in oocysts counts between days for the same individuals during the five days of the study. Finally, these authors compare caecal and intestinal types of faeces. The authors state that in many avian species caecal faeces comprise a significant proportion of total faeces excreted and can be easily and unambiguously distinguished from intestinal faeces by their colour, texture and odour (Villanúa et al., 2006). They found that, despite caecal faeces containing more oocysts, the circadian shedding pattern appears the same in each faecal type.

1.5.1.2. Circadian variation in oocyst shedding in Isospora spp.

Compared to the dearth of information on the circadian variation in oocyst output for *Eimeria* spp., this phenomenon is well described in a range of *Isospora* spp. Again, Boughton was the first to describe this phenomenon for *Isospora* spp. with the first report of it being in English sparrows (*Passer domesticus*), with peak shedding occurring between 3pm and 8pm (Boughton, 1933). Further to describing its existence, Boughton investigated possible triggering and control mechanisms by altering the light regimes for captive English sparrows (Boughton, 1933; Boughton et al., 1935). Intriguingly, when the light regime was artificially reversed the peak oocysts shedding for sparrows changes to between 3am and 8am after 3-5days (Boughton, 1933). This finding suggests that oocyst shedding is directly dependent on the photoperiod by showing a change in oocyst output in response to a change in photoperiod (Boughton, 1933; Boughton et al., 1935).

More recently, Dolnik (1999a,b) investigated circadian variation in oocyst output for *Isospora* spp. across several passerine host species. Dolnik (1999a) showed a peak in oocyst output between 4pm and 8pm, with few oocysts shed in morning samples. However, no samples were collected at 6pm, meaning inference for this time slot is not possible (Dolnik, 1999a). The author also kept a single starling in a cage over three days and found it did not excrete any oocysts between midnight and 2pm. However, with only one bird investigated results are not conclusive. As part of the same research, Dolnik (1999b) described the diurnal shedding of oocysts in six species of passerines. The author found that, although some variation occurred between individual *Isospora* spp., they all showed a peak between 4pm and 8pm with few oocysts shed in the morning samples. During the course of this research the author kept 14 birds from four species in cages to facilitate night time sampling. The author reports that very few oocysts were excreted during the morning or night time. Interestingly, the peak in oocyst shedding persisted slightly longer for these caged birds, occurring from 1pm to 9pm, compared with their wild counterparts (Dolnik, 1999b). This may suggest that captive birds shed oocysts for slightly longer than wild birds. Further to this, Dolnik (1999b) found oocysts counts are higher on the first day post-capture than the following days in captivity. It is plausible that this increased shedding is a response to the initial stress of being caught, however the author makes no suggestion as to possible reasons. There is no evidence of any statistical analysis for Dolniks (1999a,b) research. For example, although in both of these studies birds from various ages and various flocks were sampled across time (Dolnik, 1999a, 1999b), the author does not discuss the statistical analysis and it is unknown whether these possible confounding factors were accounted for. Further to this, the author does not appear to account for the different number of samples collected at different times of the day. However, this research was originally published in Russian and it is possible the English summaries omit some details.

Many other authors have also investigated this phenomenon for *Isospora* spp. across a range of host species. The majority of these have described considerable variation in

the abundance of oocysts as well as the presence or absence of oocysts (prevalence), depending on the time of sample excretion. A general trend across the literature is apparent, with few or no oocysts being shed in the morning or overnight and shedding increasing from midday through to a peak in the afternoon (Berto et al., 2011; Brawner III & Hill, 1999; Brown et al., 2001; Coelho et al., 2013; Dolnik et al., 2011; Filipiak et al., 2009; Lindström et al., 2009; Lopez et al., 2007; Misof, 2004; Morin-Adeline et al., 2011). Morin-Adeline et al. (2011) found it was more likely for oocysts to be found in afternoon samples than morning samples by a factor of 42 times. Further to this, mean oocysts counts were approximately 200 times higher in afternoon counts than morning counts in this study (Morin-Adeline et al., 2011). Filipiak et al. (2009) found that no oocysts were shed during the night and morning with oocyst counts increasing from midday to reach a peak between 5pm and 7pm. Brawner & Hill (1999) found that the number of oocysts excreted, as well as the presence or absence of oocysts, was dependent on the time of faecal excretion, with a general increasing trend from 8am through to 7pm (Brawner III & Hill, 1999). Dolnik et al. (2011) found that prevalence peaked at 4pm, however oocysts were not counted so no conclusions on abundance are possible. Lindstrom et al. (2009) found that oocysts were shed almost exclusively in the afternoon with less than 1% of morning samples being positive. This research was as part of a wider parasite survey and didn't exclusively target the circadian periodicity of oocyst shedding. As such 192 samples were collected in the morning compared with only 19 in the afternoon (Lindström et al., 2009). Lopez et al. (2007) concluded that oocyst prevalence peaked in the afternoon and dropped before the evening whereas abundance peaked during the entire afternoon. As well as the circadian variation in oocyst shedding, the authors reported that abundance varied with the host species but prevalence did not vary with either age or host species. The authors also found that even heavily infected individuals didn't shed oocysts in the morning sampling time period. Misof et al. (2004) showed that there is a greater likelihood of finding oocysts in afternoon samples as well as an increased likelihood of higher counts as birds shed oocysts more frequently and at a higher abundance in the afternoon. Finally, Coelho et

al. (2013) found that a higher proportion of faecal samples were positive for coccidial oocysts in the afternoon compared with the morning and the number of oocysts were higher in the afternoon, showing that oocysts were more likely to be shed and more likely to be found at higher numbers in the afternoon. As a result of their findings, many of these authors conclude that collecting samples from non-shedding times is unsuitable for determining coccidial prevalence or abundance and accurate testing requires sample collection at an appropriate time of the day (Brawner III & Hill, 1999; Brown et al., 2001; Coelho et al., 2013; Lopez et al., 2007; Misof, 2004). The findings of Brown et al. (2001) in particular show the potential for false negatives if time of day is not considered during sampling. These authors (2001) found that 99% of samples collected between 7am and 12pm were negative for oocysts, yet approximately one third of the birds were recaptured in the afternoon and had changed from negative to positive (Brown et al., 2001). It should be noted that the term false negative is used to refer to no oocysts being present in the faeces despite a current coccidial burden, thus falsely indicating absence of infection despite current infection. This differs to the usual use of this term to refer to a test failing to detect the presence of oocysts in a sample. This terminology is consistent with some previous authors discussing the same subject (Villanúa et al., 2006).

Although in agreement that the likelihood of an individual shedding coccidia is dependent on the time the samples are produced, Hudman et al. (2000) concluded that individuals more commonly shed oocysts during the night than the day, conflicting with other author's findings. The authors go on to recommend future studies of coccidia focus on samples collected at night time, which appears misleading in light of other research. This may highlight potential differences in the oocyst shedding pattern between coccidia species and alludes to the importance to describe this pattern for each particular *Eimeria* spp. to enable accurate interpretation of oocyst counts. However, a possible explanation is that this study only compared two time slots, 3pm to 5am as 'night' and 5am to 3pm as 'day', and variation within these time slots cannot be determined. It is likely that the peak occurs at the same time as described by other

authors, in the late afternoon with little or no shedding occurring at night time (Coelho et al., 2013; Filipiak et al., 2009; Lopez et al., 2007), yet this afternoon peak was classified as night time due to the choice of the two time slots. Further to this, the research only assessed presence or absence of oocysts meaning that only conclusions on prevalence, and not burden, are possible. Thus Hudman's et al. (2000) conclusions and recommendations may be misleading and could be disregarded now that a more detailed understanding of general oocyst shedding patterns is available (Berto et al., 2011; Brown et al., 2001; Filipiak et al., 2009; Lopez et al., 2007).

1.5.1.3. Circadian variation in oocyst shedding for nocturnal host species

There are very few reports of circadian variation in oocyst output occurring in nocturnal hosts, and the current study on kiwi is likely to be the first report in an avian host species. There is a single report of this phenomenon occurring in a nocturnal mammal, the deer mouse (*Peromyscus maniculatus*) (Fuller, Hefner, & Wrosch, 1995). This study investigated two species of *Eimeria* affecting these deer mice via artificial infection of two separate groups of mice, followed by sampling every four hours. The exact times of these samples is not given, however results are discussed with regard to the light period, which is shown to cover 16 hours, and the dark period, covering eight hours. Both species of *Eimeria* exhibited circadian variation in oocyst shedding. *E. arizonesis* peaked during the dark phase, while *E. delicata* showed four peaks across two days at 12-16 hour intervals. This appears to be a unique finding with no other reports of a coccidia species exhibiting two peaks of oocyst output within a 24-hour period encountered across the literature. Interestingly, Fuller et al. (1995) reports that for both *Eimeria* spp., oocyst concentration per faecal pellet was higher during daylight hours but that more total oocysts were shed during the night. However, although each daytime faecal pellet contained higher concentrations of oocysts than night time pellets, less faeces were produced during the day so the total oocyst shed during the day is lower. This is an unusual approach as oocyst counts are universally reported in oocysts per gram rather than total oocyst output over a period of time. If the authors had reported their results in oocysts per gram, it is possible they would have obtained

different results, depending on the difference in faecal output between time slots. Further to this, the light period is twice the length of the dark period, and it is not stated if these time differences were accounted for when calculating total oocyst output. In addition, the total number of samples for each time period is not given and it is unknown if there was equal representation across times. The authors do not give the proportion of samples that were positive for oocysts so no inference on prevalence across time may be made. Further evidence that the light/dark period may play an important role in the circadian variation of oocyst shedding is shown by early work by Boughton (1933, 1935). These studies showed that reversing the light regime resulted in a reversal of the oocyst shedding pattern for sparrows. Thus it is plausible that nocturnal animals may therefore show the opposite pattern of oocyst shedding compared to diurnal species.

1.5.2. Adaptive reasons for circadian variation of oocyst shedding

Across various families of birds, different coccidia species and multiple geographical zones, the emergence of oocysts is synchronised with a peak in the afternoon (Berto et al., 2011; Dolnik et al., 2011) suggesting strong and consistent selection pressures for this adaptation. From an evolutionary perspective anything that increases the chance of successful transmission, and therefore subsequent reproduction, of the coccidia would be selected for (Dolnik et al., 2011). The successful transmission of coccidia relies on both the successful sporulation and survival of the oocysts followed by successful ingestion by an appropriate host (Dolnik et al., 2011). Therefore circadian periodicity is likely to be an adaptation of coccidia to increase successful sporulation and survival of oocysts and/or to increase the probability of being ingested by a susceptible host (Dolnik et al., 2011; Martinaud et al., 2009). Although the adaptive reasons for circadian rhythm in coccidia are still unclear and the underlying reasons for this periodicity of oocyst shedding are not understood (Brown et al., 2001; Dolnik et al., 2011), two main hypothesis have been proposed to explain the circadian variation in oocyst shedding (Berto et al., 2011; Dolnik, 1999a, 1999c; Martinaud et al., 2009; Misof, 2004).

1.5.2.1. Increased ingestion hypothesis

The first hypothesis suggests that the peak in oocyst shedding in the late afternoon by multiple species of coccidia affecting avian hosts is an adaptation to increase the chance of oocysts being ingested by susceptible conspecifics (Berto et al., 2011; Dolnik, 1999a; Dolnik et al., 2011; Lopez et al., 2007). Afternoon shedding coincides with a time of high feeding activity during which many species of birds congregate in common feeding areas (Berto et al., 2011; Dolnik et al., 2011; Martinaud et al., 2009). Oocysts shed during this feeding time will likely be excreted onto the communal feeding areas and thus have a better chance of subsequent ingestion by, and transmission to, susceptible conspecifics (Berto et al., 2011; Dolnik, 1999a; Dolnik et al., 2011; Martinaud et al., 2009). Although oocysts are shed unsporulated and are therefore not immediately infective, Martinaud et al. (2009) found most *Isospora turdi* oocysts shed in the late afternoon by black birds (*Turdus merula*) were infective by the following morning, meaning birds feeding in the same area will be exposed to these infective oocysts the day after they were excreted.

However Dolnik et al. (2011) found circadian variation in oocyst shedding exists in snow buntings (*Plectrophenax nivalis*) in the Arctic where constant daylight results in a lack of consistent diurnal foraging behaviour patterns. In this situation there is no increased chance of exposure to conspecifics by being shed at a particular time, yet the oocyst shedding pattern still exists, questioning this hypothesis. Further to this, most bird species also have a period of equally high feeding activity in the morning (Berto et al., 2011; Coelho et al., 2013; Martinaud et al., 2009) and the hypothesis that releasing oocysts in the late afternoon increases the probability of encounters between host and parasite fails to explain why oocysts are not also shed during the morning feeding hours, where the same principles would apply with regard to access to conspecifics. This may be explained in migratory species where close to migration they cease to feed in the morning meaning the afternoon is the most reliable time for oocysts to be shed (Dolnik, 1999b). Lopez et al. (2007) found that two species of passerines with different feeding patterns still showed the same oocyst shedding pattern, and this result

suggests oocyst shedding is not directly related to feeding activity. Finally, Berto et al. (2011) suggests that in tropical areas, if oocysts were excreted during the morning, many would be washed from feeding areas by heavy daytime rains before they have sporulated and been exposed to vulnerable conspecifics.

1.5.2.2. Decreased desiccation hypothesis

The second hypothesis suggests that the peak in oocyst shedding in the late afternoon is an adaptation to prevent the desiccation of unsporulated oocysts in order to increase their chance of survival in the environment (Berto et al., 2011; Coelho et al., 2013; Filipiak et al., 2009; Martinaud et al., 2009). Successful transmission of coccidia between individuals requires the survival and sporulation of oocysts in the environment to facilitate infection of susceptible hosts. Unsporulated oocysts are particularly vulnerable to desiccation, which can kill or reduce the infectivity of oocysts. However once sporulated, oocysts become more resistant to these environmental threats (Berto et al., 2011; Dolnik, 1999c; Dolnik et al., 2011; Martinaud et al., 2009). Thus oocyst survival and sporulation success are increased by shedding during the afternoon as it enables the oocysts to sporulate overnight, becoming much more resistant to desiccation through exposure to sunlight the following day (Dolnik et al., 2011; Martinaud et al., 2009). If oocysts were shed constantly throughout the day many would die from desiccation due to exposure to sunlight before having the chance to sporulate, and the release of oocysts in the late afternoon avoids this low survival rate (Berto et al., 2011; Coelho et al., 2013; Martinaud et al., 2009).

This theory has been supported by the findings of two recent studies. Firstly, Martinaud et al. (2009) found that exposure to sunshine dramatically reduced oocyst viability, with one hour of exposure reducing viability by about 50% and after four hours few oocysts remained viable. With further investigation the authors were able to determine that this effect is mainly due to ultraviolet (UV) radiation and high temperature caused by sunlight (Martinaud et al., 2009). Secondly, investigations of Arctic snow buntings revealed they still exhibit diurnal variation in oocyst shedding despite not having consistent foraging activity patterns, suggesting there is no

increased chance of exposure to conspecifics by being shed at a particular time (Dolnik et al., 2011). However the UV light level in the Arctic does still vary by as much as five times within a 24-hour period, suggesting that it is the variation in UV levels that has led to the observed diurnal shedding in order to avoid desiccation rather than the access to conspecifics at feeding time (Dolnik et al., 2011). However it is also possible that the diurnal shedding rhythm is set at the snow buntings wintering grounds in the temperate zone where a relatively normal dark-light pattern, as well as the corresponding feeding patterns, exists and that the rhythm persists during the hosts breeding season in the Arctic (Dolnik et al., 2011). Further to this supporting evidence, sporulation takes between 18 hours and four days for *Isospora* spp. affecting passerines (Dolnik, 1999c), and 12 to 30 hours for *Eimeria* spp. affecting chickens (McDougald, 2003), and this timeframe is probably sufficient for the majority of oocysts to sporulate overnight before exposure to damaging levels of UV the following day.

1.5.2.3. Combination of both hypotheses

Ultimately it is likely that the release of oocysts in the late afternoon enhances both the survival and transmission of the parasite (Martinaud et al., 2009) but the exact adaptive reasons for this circadian rhythm in coccidia are still unclear (Dolnik et al., 2011). It seems probable that the adaptive significance of diurnal shedding is a combination of both current hypotheses with afternoon shedding increasing the chances of contact between host and parasite by occurring at peak feeding time, as well as ensuring maximum survival and sporulation by allowing oocysts time to sporulate before exposure to sunlight (Dolnik, 1999b; Martinaud et al., 2009).

1.5.3. Possible triggering mechanisms for oocyst shedding

A similar pattern of diurnal periodicity of oocyst shedding is seen across a broad range of avian species and it is therefore likely that the reasons for this adaptation are the same across these different host species. It is therefore also assumed that the underlying mechanisms triggering this diurnal pattern are the same across all host species. Although many authors can agree on the possible underlying adaptive reasons

for the development of diurnal shedding, little is known about the possible underlying physiological, anatomical or developmental mechanisms that trigger oocyst shedding (Lopez et al., 2007; Misof, 2004). There are several current theories, many of which are plausible but none of which are proven, and there is little consensus in the literature.

In general the adaptive value of a circadian rhythm is the advantage for the organism in synchronising its behaviour and physiology with the periodically changing environment and for parasites, these environmental changes are experienced via their hosts (Dolnik et al., 2011). This means that increased oocyst output in the afternoon is likely to be an adaptation to the physiological rhythms and behaviour of the host (Dolnik, 1999a). Thus it is likely that the triggering of oocyst shedding is controlled by an aspect of the host's physiology (Coelho et al., 2013; Lopez et al., 2007). Lopez et al. (2007) suggests feeding habits may have an effect on oocyst elimination patterns, such as increased host activity leading to shedding. However, it has been shown that host temporal patterns of feeding do not directly affect the temporal pattern of oocyst shedding, suggesting that increased host activity does not act as a direct triggering mechanism for oocyst shedding (Boughton, 1933; Dolnik et al., 2011). Dolnik's et al.(2011) research showed that during the Arctic summer the diurnal oocyst shedding pattern persists in snow buntings, despite the host exhibiting no consistent diurnal feeding pattern in the constant light. Further to this, Boughton (1933) found that when the feeding times of house sparrows were altered the oocyst shedding pattern remained constant and Lopez et al. (2007) found that two species of passerine with different feeding patterns still had similar oocyst shedding patterns. Finally, most bird species also have a period of equally high feeding activity in the morning (Berto et al., 2011; Coelho et al., 2013; Martinaud et al., 2009; McQuistion, 2000) and if increased activity was the triggering mechanism we would expect to see increased oocyst shedding at this time also.

As altering light patterns will also alter the circadian rhythm of oocyst shedding (Boughton, 1933; Dolnik et al., 2011), it has been suggested that the corresponding cyclic changes in melatonin may play a role in triggering oocyst release rhythms (Dolnik

et al., 2011; Filipiak et al., 2009). Melatonin is important for the regulation of circadian rhythms in birds and its production in the pineal gland is initiated by darkness and inhibited by light (Dolnik, 1999a; Saper, Scammell, & Lu, 2005). As melatonin is responsible for diurnal activity in some other parasite genera, such as *Plasmodium* spp. (Bagnaresi, Nakabashi, Thomas, Reiter, & Garcia, 2012), it seems a logical triggering mechanism for the circadian periodicity of oocyst shedding. It has also been suggested that the effect of melatonin on the activity of the avian immune system may indirectly lead to increased oocyst shedding in the afternoon (Dolnik, 1999a). However, although artificially altering the melatonin levels in birds can break down their circadian rhythms, it does not appear to synchronise oocyst output rhythms for *Isospora* spp. ((Brandlmeier) as cited by Dolnik, 2011). During constant daylight, at least some avian species exhibit constant levels of melatonin (Miché et al., 1991). However, even under these conditions, the diurnal pattern of oocyst shedding has been sown to persist in some host species, even in the absence of constant foraging patterns (Dolnik et al., 2011). In this case, coccidia are unable to use either their host's melatonin levels or foraging activity to set an oocyst excretion rhythm as both are constant, yet a peak output still occurs in the late afternoon suggesting there are other triggering mechanisms occurring (Dolnik et al., 2011).

Anatomy has also been suggested to play an important role in the pattern of oocyst shedding. Defecation of the colonic and caecal contents have been shown to occur at varying ratios throughout the day, with additional variation between avian species (Misof, 2004; Williams, 1995). This may lead to circadian variation in the detection of coccidia species that colonise different parts of the intestines (Williams, 1995). This may be further complicated in kiwi by peristaltic caecal contractions, in conjunction with colonic antiperistaltic contractions, which may extrude the fluid component of colonic digesta along with suspended fine particulate material (Potter et al., 2006) resulting in convoluted and poorly understood gut passage patterns. However, Villanua et al. (2006) found that, despite varying levels of oocysts in intestinal and

caecal faeces, the circadian shedding pattern was the same for both faecal types for *Eimeria* spp. affecting partridges.

As well as this, the variation in the amount of faeces produced at different times of the day may influence the appearance of a diurnal rhythm (Lopez et al., 2007). However, Boughton (1937) found variation in shedding of oocysts despite consistent faecal volumes throughout the day in caged pigeons, suggesting the diurnal pattern of oocyst shedding is unrelated to faecal output.

As findings in some studies suggest that neither melatonin nor increased host foraging activity are responsible for triggering oocyst shedding (Dolnik et al., 2011), and there is scant evidence supporting other suggested triggers, the mechanisms behind the observed diurnal oocyst shedding patterns currently remain unknown.

1.5.4. Implications of diurnal periodicity of oocyst shedding

Estimation of the intensity of a coccidia burden is essential to investigate the impact of parasitism on a population and the analysis of faecal material for parasitic stages, such as oocysts, is commonly used as a diagnostic test for parasite burdens (Coelho et al., 2013; Filipiak et al., 2009; Misof, 2004). However the circadian periodicity of oocyst shedding is an important consideration when sampling birds for faecal oocyst counts as the time of day at which samples are collected can drastically affect the results of parasite assessments (Brawner III & Hill, 1999; Dolnik, 1999b). Only faeces collected during shedding times will reflect the true parasite prevalence and sampling outside of the shedding time may indicate absence of infection despite current infection (Brown et al., 2001; Lopez et al., 2007; Morin-Adeline et al., 2011). As such, estimates of coccidia prevalence and abundance that do not take the time of day into consideration are unreliable (Boughton, 1937c; Brawner III & Hill, 1999; Coelho et al., 2013; Misof, 2004) and have been shown to be inaccurate for many species due to the variable shedding of oocysts throughout the day (Brawner III & Hill, 1999; Brown et al., 2001; Lopez et al., 2007; Morin-Adeline et al., 2011).

As the circadian rhythm of shedding is likely to be the result of long term co-evolution between the parasite and the host, and as selective pressures differ between host species so too will the oocyst shedding cycle (Filipiak et al., 2009). Therefore researchers must develop an understanding of the shedding pattern for each coccidia species as well as any other host-parasite interactions in order to be able to accurately assess the parasitic load (Filipiak et al., 2009).

As the time of day that samples are collected has a significant effect on the reliability and accuracy, and therefore the validity, of the resultant data (Brawner III & Hill, 1999; Misof, 2004), an understanding of the shedding pattern for a particular species of coccidia across time is required to enable meaningful interpretation of oocyst counts (Filipiak et al., 2009). In order to make accurate comparisons of coccidial loads between individuals, samples must be collected from the same time of day for all birds as only a few hours difference may have a significant effect on the reliability and validity of the resultant data (Filipiak et al., 2009; Lopez et al., 2007). As well as this, the potential variation of oocyst shedding between successive days for an individual further compromises the ability to accurately compare counts between individuals or populations and repeated measurements of several individuals across multiple days may be necessary to arrive at reliable conclusions (Filipiak et al., 2009). Although some studies have shown significant variation in oocyst counts between days (Dolnik, 1999a; Filipiak et al., 2009; Villanúa et al., 2006), other authors report consistently comparable oocyst counts between days (Boughton, 1937a; Dolnik, 2006; Morin-Adeline et al., 2011) suggesting that this may vary between host and parasite species. Further to this, many host species are susceptible to concurrent infection with several species of coccidia, with these mixed infections possibly further complicating oocyst shedding patterns due to the potential for different diurnal shedding patterns between species (Filipiak et al., 2009; P. P. Levine, 1942; Morgan et al., 2017).

This potential inability to accurately assess parasite burdens due to diurnal oocyst shedding can lead to consequences such as false validation or rejection of hypotheses

in relation to parasite infections. For example developing hypotheses on the relationship between sexual selection by birds based on diminished displays of ornamentation due to parasite burdens rely on accurate assessment of parasite burdens (Brawner III & Hill, 1999). As well as this, inaccurate estimates of coccidia burdens may have a direct impact on management decisions with regard to coccidia control. This is particularly important when health screening captive or wild birds as sampling from the wrong time may give false negative results, even for heavily infected individuals (Brown et al., 2001; Lopez et al., 2007; Morin-Adeline et al., 2011). This may result in poor management decisions, such as facilitating the spread of coccidia by releasing infected birds into potentially uninfected or naïve populations. Ultimately, to gain accurate insights into a population or an individual's coccidial burden via faecal oocyst counts, the circadian variation in oocyst shedding must be understood and accounted for.

1.6. Methodology

1.6.1. Sample collection method

Various different methods have been used across the literature to investigate the circadian variation of oocyst shedding in various avian species (Table 2). As much of the research on circadian variation has been carried out on wild species, the previously used methodologies have been limited by access to birds. Some authors have relied on opportunistic sample collection during handling for other reasons (Coelho et al., 2013; Lindström et al., 2009); some specifically captured birds for sampling (Brawner III & Hill, 1999; Brown et al., 2001; Dolnik, 1999a, 1999b; Filipiak et al., 2009; Hudman et al., 2000; Lopez et al., 2007; Martinaud et al., 2009); others worked with captive individuals (P. P. Levine, 1942; Morin-Adeline et al., 2011; Villanúa et al., 2006); and some studies observed wild birds without disturbing them (Dolnik et al., 2011; Misof, 2004). The previously used methodologies can be divided into two broad groups; firstly specific and detailed data from following trends in a smaller number of individuals (eg (Brawner III & Hill, 1999; Martinaud et al., 2009; Villanúa et al., 2006)), and secondly

data from large numbers of samples from many, unspecified, individuals following trends across all samples (eg (Dolnik et al., 2011; Lindström et al., 2009; Lopez et al., 2007; Misof, 2004))

1.6.1. Oocyst counting technique

A large number of different quantitative microscopic methods and modifications are reported for counting oocysts (Table three), with the McMaster technique, developed at the McMaster laboratory of the University of Sydney, generally the most commonly used (Silva et al., 2013; Vadlejch et al., 2011). However, a limitation of the McMaster counting chamber is that its thickness does not allow the use of high powered (>20x) objectives, preventing the accurate morphological identification of oocysts. A relatively new method of oocyst counting is the mini-FLOTAC device (Cringoli et al., 2017; Cringoli, Rinaldi, Albonico, Bergquist, & Utzinger, 2013). The mini-FLOTAC does not require centrifugation of samples, is easy to use, cheap, and not overly time consuming. This device also offers several advantages over the McMaster chamber including the presence of guidelines on the reading disc to facilitate accurate counting and clearer visibility (Silva et al., 2013), as well as being thin enough to allow the use of an 40x objective. The mini-FLOTAC has been shown to have higher sensitivity for detecting parasite eggs than other diagnostic techniques (Barda et al., 2014; Cringoli et al., 2017; Rinaldi et al., 2014; Silva et al., 2013) and also has high accuracy and precision (Cringoli et al., 2017). Silva et al. (2013) compared the mini-FLOTAC to the McMaster technique for the diagnosis of *Eimeria* spp. in goats and found mini-FLOTAC to be a sensitive, accurate and easy to perform technique. The author also reported higher oocyst counts per gram with the mini-FLOTAC compared to the McMaster technique.

There are a number of solutions used for the flotation of oocysts. Ryley et al. (1976) found that oocyst recovery was similar, and even slightly better, for saturated salt solution when compared to zinc sulphate or sucrose solution (Ryley, Meade, Hazelhurst, & Robinson, 1976), adding to the important factors of ease of making, cheapness and safety.

Table 2

A summary of previous methods used to investigate and describe the circadian variation in oocyst shedding for species of coccidia affecting various avian host species.

Authors	Method Summary	Number of hosts sampled (total samples)	Host captured +/- Caged	Duration	Time slots	Sampled during night time	Presence or Abundance	Follow individual birds' patterns	Know exact time of excrement	Oocyst counting method/ flotation solution
Misof, 2004	Watched wild birds and collected droppings over 3 years	57 (114)	No	Summer of 3 years	7am-12pm 12pm-6pm	No	Abundance	No	Yes	Mcmaster/ Saturated salt soln.
Lopez et al., 2007	Caught in mist nests and held in bags for 20mins to collect samples, separated urine and intestinal components and only analysed intestinal	406 (406)	Yes	Spring of 2 years	Grouped by 1hour	No	Abundance	No	Yes	Mcmaster/not stated
Villanua et al., 2006	Separated infected captive birds in cages with mesh floors, put fresh paper in 5 minutes before scheduled time and collected first dropping	6 (167)	No	1 month	8am, 12pm, 4pm, 7.30pm	No	Abundance	Yes	Yes	McMaster/ Zinc sulphate
Levine, 1942	Infected young chickens with oocysts from different species of *Eimeria*, collected samples from each group at 9am,3pm and 9pm.	50 (*not stated*)	No	1 week	9am-3pm 3pm-9pm 9pm-9am	Yes	Abundance	No	No	Not stated
Brawner & Hill,	Captured birds before 10am and after 3pm and collected	26 (26)	Yes	1 day	Before 10am,	No	Presence	No	Yes	Float/ Sheathers

1999 Part 1.	sample at time of capture				after 3pm					soln.
Brawner & Hill, 1999 Part 2.	Captured wild birds and caged. Paper lining replaced every 4 hours from 8am-7pm and first sample for each time slot used.	8 (*32*)	Yes + caged	2 months	4 hourly	No	Abundance	Yes	Yes	Float/ Sheathers soln.
Brown et al., 2001	Captured birds and collected samples at time of capture	404 (404)	Yes	5 months	Grouped by 1 hour	No	Presence	No	Yes	Float/ Saturated salt soln.
Dolnik, 1999b Part 1.	Caged individual birds for several days and the paper changed in bottom of cage every 2 hours.	14 (*not stated*)	Yes + caged	1 week	2 hourly	Yes	Abundance	Yes	No	Float/ Saturated salt soln.
Dolnik, 1999b Part 2.	Captured birds from various species, collected samples at time of capture	362 (362)	Yes	3 years	Grouped by 1 hour	No	Abundance	No	Yes	Float/ Saturated salt soln.
Dolnik, 1999a	Captured birds and caged until sample excreted then released	100 (100)	Yes + caged	Summer of 3 years	Grouped by 1 hour	No	Abundance	No	Yes	Float/ Saturated salt soln.
Coelho et al.,	Collected samples from birds kept in quarantine following recovery from trafficking, cleaned cage and collected all droppings between 2 time slots	164 (220)	No	7 months	9am-12pm and 3pm-5pm	No	Abundance	Yes	No	Float/ Sheathers soln.
Dolnik at el., 2011	Observed birds and collected droppings when seen across 24 hours	100 (250)	No	July of 3 years	Split into 2 hourly slots	Yes	Presence	No	Yes	Float/ Saturated salt soln.
Filipiak et al., 2009	Captured birds and kept in individual cages. Cages	10 (80)	Yes + caged	2 months	Overnight then 2	Yes	Abundance	Yes	No	Mcmaster/ Sheathers

	cleaned at ~8pm and first sample taken at 8am (ie overnight samples) then samples removed every 2 hours during the day for 1 day.				hourly during day					soln.
Morin-Adeline et al., 2011	Collected all droppings done between 8am-11am and 2.30-5.30pm from the floor of aviaries containing captive birds on 2 days	53 (174)	No	2 days	8am-11am and 2.30-5.30pm	No	Abundance	No	No	Mcmaster/ Saturated salt soln.
Lindstrom et al., 2009	Captured 400 birds and put in paper bags until processing, opportunistically collected droppings	219 (219)	Yes	3 months	Before 12pm, after 12pm	No	Presence	No	Yes	Float/ Zinc sulphate
Hudman et al., 2000	Captured and caged birds, collected all droppings from between 5am-3pm and 3pm-5am	67 (108)	Yes + caged	Summer of 2 years	5am-3pm, 3pm-5am	Yes	Presence	No	No	Float/ Sheathers soln.
Boughton, 1937c	Captured birds and kept in cages with wire mesh floor for 7 days, used paper to collect samples from 3 time slots	5 (*not stated*)	Yes + caged	1 week	9am-3pm 3pm-9pm 9pm-9am	Yes	Abundance	Yes	No	Float/ not stated

Table 3

Characteristics (diagnostic performance and technical performance) and main limitations of different copromicroscopic techniques used for the diagnosis of protozoan infections. Modified and used with permission from Cringoli et al. (2017).

Technique	Diagnostic performance			Technical performance			
	Sensitivity	**Accuracy**	**Precision**	**Cost**	**Processing time**	**Equipment needs**	**Main limitation**
Float	Very low	Very low	Very low	Inexpensive	Medium	Basic laboratory equipment	Lack of precision, owing to the absence of a grid on the coverslip
McMaster	Medium	Low	Low	Expensive	Medium	Basic laboratory equipment	Inability to use high powered objectives (>20x) due to thickness
Kato-Katz	Medium	Medium	Low	Inexpensive	Long	Basic laboratory equipment	Requires fresh droppings
FLOTAC	Very high	Very high	Very high	Inexpensive	Long	Fully equipped laboratories	Requires centrifugation steps with two different rotors and so an equipped laboratory
Mini-FLOTAC	High	High	High	Inexpensive	Medium	Basic laboratory equipment	Detection of some parasites (e.g., trematoda) requires centrif-ugation and so, for very accurate applications, it is suggested that the FLOTAC technique be used

1.6.2. Specific considerations for investigating circadian variation in oocyst shedding

The majority of previous researchers investigating circadian variation in oocyst shedding used local time (Coelho et al., 2013; Misof, 2004; Morin-Adeline et al., 2011), however Lopez et al. (2007) standardised the time of sample collection based on hours after sunrise. This standardisation is a novel approach and was carried out due to the large variation in daylight length during the sampling period. This variation of two hours and 36 minutes meant that hourly data from different days would not be comparable (Lopez et al., 2007). As day length varies throughout the year and between locations, this standardisation may allow direct comparison of results from research from different geographical areas or different times of year. However if the aim is to define a species oocyst shedding pattern for a particular geographical location at a certain time of year then comparison with other research is not a priority and the simpler, more practical, approach of using the time of day may suffice. Further to this, real time is likely to be more useful from a management perspective than standardised time.

Dolnik (1999b) found that oocyst shedding from caged birds persisted for longer than their wild counterparts and that oocyst counts are higher during the first day of captivity following capture (Dolnik, 1999a). This suggests that capturing birds may have a direct effect on oocyst shedding, possibly due to stress. Several previous authors captured and restrained wild birds in order to obtain samples (Brawner III & Hill, 1999; Brown et al., 2001; Dolnik, 1999a, 1999b; Filipiak et al., 2009; Hudman et al., 2000; Lindström et al., 2009; Lopez et al., 2007; Martinaud et al., 2009) or sampled captive birds (Coelho et al., 2013; P. P. Levine, 1942; Morin-Adeline et al., 2011; Villanúa et al., 2006), with few authors collecting samples without disturbing the birds (Dolnik et al., 2011; Misof, 2004). None of these authors addressed the possibility of this affecting their results, with the exception of Villanua (2006) who suggests stress likely results in increased oocyst counts. However as all birds within each study underwent the same treatment it is unlikely to affect the intra-study oocyst shedding pattern. Further to

this, all studies appear to show very similar oocyst shedding patterns despite the various different methods, including observation only studies, suggesting that capturing birds will not considerably affect the circadian variation in oocyst shedding.

Oocyst counts are universally reported as 'oocysts per gram' of sample, thus a potential confounding factor in the description of the circadian variation in oocyst shedding is the potential for faecal volume or consistency to influence oocyst count results. Some previous researchers accounted for the potential effect of varying faecal consistency. Lopez et al. (2006) carried out oocyst counts and then subsequently dried the diluted, homogenised sample and weighed it to the nearest 0.0001g. This allowed results to be expressed as oocysts per milligram of dry extract of faeces. Misof et al. (2004) and Morin-Adeline et al. (2011) centrifuged each sample, removing the supernatant and weighing the pellet before suspending it in flotation medium. However, the majority of researchers made no attempt to control for this potential confounder (Brawner III & Hill, 1999; Coelho et al., 2013; Dolnik, 1999a, 1999b; Dolnik et al., 2011; Filipiak et al., 2009; P. P. Levine, 1942; Lindström et al., 2009; Villanúa et al., 2006). Further to this, variation in the amount of faeces produced at different times of the day may influence the diurnal rhythm of oocyst shedding (Lopez et al., 2007). However, Boughton (1937) found the volume of faeces excreted by the pigeons was uniform throughout the day and concluded that the variation in oocyst shedding could not therefore be explained simply by variation in faecal output. This concurs with his previous findings that higher oocyst counts do not occur simply due to larger faecal volumes (Boughton et al., 1935). Further to this, it appears that the differences in oocyst counts across time are too large to be explained by variation in faecal volume or consistency alone. For example Morin-Adeline et al. (2011) found a difference in oocyst counts of 200x between shedding and non-shedding and it is unlikely that faecal volume or consistency could vary by enough to explain this alone.

Droppings from avian species contain both intestinal components and renal components (urine and urates). Lopez et al. (2006) separated the intestinal and renal

portions of the droppings to avoid the urates affecting the mass of the sample. This technique is not repeated across the literature. Similarly to the variation in faecal consistency or volume, the presence or absence of urates is unlikely to have enough of an effect on dropping weight to explain such large variation in oocysts counts as has been reported. Further to this, it is not always possible to easily distinguish and separate the renal and intestinal components of kiwi droppings (*personal observation*). Finally, ratite ureters open directly into the coprodeum which may facilitate the reflux of urine into the large intestine (C. A. Oliveira, Silva, Santos, & Mahecha, 2004; Potter et al., 2006). If this anatomy is consistent for kiwi, it is possible that the intestinal and renal components of the dropping have already mixed to some extent before being excreted, meaning separation of renal and intestinal components would achieve little.

A final consideration is the potential for oocyst shedding to vary significantly between days. Filipiak et al. (2009) found a large amount of variation in *Isospora* spp. oocyst shedding between days over a period of 10 days and Villanua et al. (2006) reports significant between-day variation in oocyst counts for *Eimeria* spp. across five days. Dolnik (1999a) also found different oocyst output across days for a single starling kept in a cage. However, Boughton (1937) found very little variation in *Eimeria* spp. oocyst output between days. This concurs with Morin-Adeline et al. (2011) and Dolnik (2006) who found *Isospora* spp. oocyst production was similar across days. Although this day to day variation may be an important consideration if wanting to compare between days, it is unlikely to have any effect on the variation within a day observed due to circadian variation in oocyst shedding.

1.7. Conclusion

With the developing understanding of parasite-host interactions, the clinical and ecological implications of coccidia parasites are yet to be fully understood even for well-studied species (Morin-Adeline et al., 2011). In order to gather accurate information on parasite burdens within kiwi populations to facilitate informed management decisions, our understanding of the host-parasite interaction must

increase. Recovery and re-introduction programmes, such as ONE, must establish baseline data for potentially threatening infectious agents and knowledge of the life history of these infectious agents is vital for the success of such programmes (Coelho et al., 2013). The high stocking rate and regular turnover of vulnerable young kiwi cycling through ONE facilities has led to an increased risk of coccidia infection and currently little information is available specifically on kiwi coccidia (Morgan, 2013). In order to ensure the on-going success and sustainability of ONE it is imperative that knowledge on the various *Eimeria* spp. affecting kiwi is improved to facilitate precise and appropriate management decisions in the future. At present, a major complicating factor in interpreting oocyst counts from kiwi droppings is the lack of understanding around coccidia behaviour with regards to the potential for circadian variation in oocyst shedding. Should this shedding exist for the coccidia species affecting kiwi it must be incorporated into the interpretation of oocyst counts on droppings or the results may be inaccurate, invalid and misleading. Thus the aim of the following research is to determine if the *Eimeria* spp. affecting brown kiwi exhibit circadian variation in oocyst shedding. If this variation is present the aim will be to define the pattern of shedding with particular focus on the time of peak shedding, as well as determine the presence of any variation between *Eimeria* spp.

Chapter Two: The circadian variation of oocyst shedding of *Eimeria* spp. affecting brown kiwi (*Apteryx mantelli*)

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2.1. Abstract

Captive rearing of wild brown kiwi (*Apteryx mantelli*) is widely carried out to assist in the recovery of this declining species. As a consequence, high densities of immunologically naïve kiwi are commonly housed in semi-captive conditions, with the potential to result in substantial morbidity and mortality from coccidiosis caused by multiple species of *Eimeria*. Previous research has described circadian variation in oocyst shedding across multiple avian host species. The aim of this research was to describe any circadian variation in oocyst shedding in brown kiwi. Droppings were

collected from brown kiwi (n=4) at a single captive rearing facility using video surveillance to determine the time of excretion, and oocyst counts were undertaken. Results show that two of the *Eimeria* spp. affecting brown kiwi exhibit a peak in oocyst shedding between 03.00 and 07.00 with few or no oocysts shed between 08.00 and midnight. These results are not able to be explained by the current hypotheses theorising the evolutionary forces behind the development of this adaptive trait. Our findings increase the current understanding of the biology of the *Eimeria* spp. affecting brown kiwi and have important implications for the management of captive-reared kiwi, in particular for the accurate interpretation of faecal oocyst counts.

Keywords; kiwi; coccidia; circadian rhythms; diurnal shedding; *Eimeria*; *Apteryx*

2.2. Introduction

Brown kiwi (*Apteryx mantelli*), a flightless, nocturnal bird endemic to New Zealand, are classified as at risk and in decline (Robertson et al., 2017). Captive rearing of young kiwi for release back into the wild is a vital tool for the short to medium term recovery of declining brown kiwi populations (Bassett, 2012; Colbourne et al., 2005; Holzapfel et al., 2008). This intensive rearing system results in a much higher stocking rate of immunologically naïve kiwi compared with a wild situation, resulting in a build-up of pathogens, in particular *Eimeria* spp. (Bassett, 2012; Doneley, 2006; Morgan, 2013). To mitigate the impacts of coccidiosis, most kiwi rearing facilities have a coccidia control programme that relies on regular oocyst counts, and subsequent appropriate administration of toltrazuril (25mg/kg PO) (Doneley, 2006; Morgan, 2008). The appropriate timing of this treatment is dependent on each facilities circumstances and often at the discretion of staff. However, these protocols do not take into account the possibility of a circadian periodicity in oocyst shedding, a phenomenon previously suggested to occur with kiwi *Eimeria* spp. (Morgan, 2013). These daily variations in

oocyst shedding have been demonstrated in both *Isospora* spp. and *Eimeria* spp. of many avian host species, with markedly different numbers of oocysts reported to be shed at various times of the day (Brawner III & Hill, 1999; Brown et al., 2001; Coelho et al., 2013; Dolnik, 1999a, 1999b; Dolnik et al., 2011; Filipiak et al., 2009; Hudman et al., 2000; P. P. Levine, 1942; Lindström et al., 2009; Lopez et al., 2007; Misof, 2004; Morin-Adeline et al., 2011; P. S. d. Oliveira et al., 2017; Villanúa et al., 2006). When present, this phenomenon limits the accuracy of parasite quantification via dropping analysis due to the potential to sample during low shedding times (Coelho et al., 2013; Filipiak et al., 2009). Consequently, if circadian variation in oocyst shedding is not accounted for, the results of such testing are unreliable and may be misleading (Coelho et al., 2013; Filipiak et al., 2009; Misof, 2004). The aim of the present study was to describe any circadian variation in oocyst shedding by *Eimeria* spp. of brown kiwi (*A. mantelli*) and to determine the management implications of this for captive reared kiwi. To our knowledge this is the first time this has been investigated in a nocturnal avian species and may have important implications that are applicable to other nocturnal species.

2.3. Method

2.3.1. Study Design

The study period ran from the 22nd of February 2017 (sunrise; 06.51 sunset; 20.04pm NZDT) to the 20th of April 2017 (sunrise; 07.47 sunset; 18.39 NZDT). A single sampling date is classified as the period from 20.00 to 19.59 the following day. Each bird sampled (n=4) was individually housed in an outside enclosure and motion detecting cameras (Ltl Acorn Trail Camera, 1080p, Acorn Cameras, Shenzen City, China) (n=7) with night filming capability were set up to enable recording of the location and time of dropping excretion. As kiwi are nocturnal, filming only occurred during times of darkness when the birds were out of their burrows and active, generally from 20.00 to 08.00. The following morning, samples were collected and assigned their corresponding time (NZDT) of excretion using video footage information. This method

is described in detail in appendix one. Assigned times were classified into one hour time slots (20.00-20.59; 21.00-21.59 etc). If the collected dropping was excreted outside of the field of camera view, or the time of dropping excrement could not be definitively determined via the camera footage, the sample was excluded. When a management procedure resulted in the kiwi being removed from its burrow the day following overnight sampling, a dropping sample was opportunistically collected from the burrow, however the time of excrement for these day samples was unknown. This research facility carries out regular testing and subsequent treatment of kiwi as necessary with oral toltrazuril (25mg/kg, Baycox^{TM} coccidiocide for piglets, 50mg/ml, Bayer, Leverkusen, Germany) and the number of days since the most recent toltrazuril administration was recorded for each sampling date.

2.3.2. Parasitology

One gram of droppings was homogenised with saturated sodium chloride solution (SG = 1.2) at a 1:20 ratio. The homogenised solution was then immediately placed into a mini-FLOTAC (University of Naples Federico, Naples, Italy) and left to stand for 10 minutes in order to facilitate flotation of oocysts. All oocysts present were counted at 200x magnification using a light microscope to give a total oocyst count (oocysts per gram, opg). This method is described in detail in appendix two. If a sample weighed less than one gram the entire sample was used and the subsequent count was adjusted to give a value as opg. In order to differentiate between the various species of *Eimeria*, an eyepiece micrometer was used to facilitate measurement of the first 25 oocysts found to provide a relative ratio for extrapolation of the total oocyst count. To determine presence and relative abundance at the study site, *Eimeria* spp. were differentiated using previously described morphological characteristics (Morgan et al., 2017). Individual results were recorded on a standardised form (appendix three) and are recorded in full in appendix four. Appendix five provides a summarised version following grouping into one hour time slots.

2.4. Statistical Analysis

The data was analysed using the Statistical Analysis System, version 9.4 (SAS Institute Inc., Cary, NC, USA). Total oocyst counts for all individual samples were transformed using a Log2 transformation in an attempt to normalise their distribution prior to analysis. Any counts exceeding 35000 oocysts per gram (opg) were excluded (n=3) due to their excessive effect on the data. Log2-transformed oocyst counts were then grouped into one-hour blocks. Least squares means and standard errors of Log2-transformed oocyst counts for each sampling hour were obtained using the MIXED procedure with a model for repeated measures. The model included the fixed effect of sampling hour (1-hour blocks) and the random effect of animal to account for repeated measures on the same animal. Days since last treatment with toltrazuril was used as a covariable with linear and quadratic effect. Significant effects were declared when the p-value was <0.05.

2.5. Results and Discussion

A total of 73 night time and nine day time samples were collected across 18 sampling dates from four individual birds (Table 4). Oocyst counts varied from 0 to 328080opg. At least one sample was positive for oocysts on 17 of the 18 sampling dates and 71% (58/82) of all samples contained oocysts. For all times between 03.00 and 07.00 the total oocyst counts were significantly higher ($p<0.05$) when compared to each time slot between 20.00 and midnight (Figure 1). This shows that high oocyst counts are dependent on time and are significantly more likely to occur between 03.00 and 07.00 than they are between 20.00 and midnight. There were no consistent statistical differences when comparing each of the time slots between midnight and 03.00, with various individual time periods being statistically significantly different to each other but no over-all trend occurring. The time slot from 07.00 to 07.59 was not significantly different to any other time slots and no trend can be determined for this time. Analysis of data collected during the day (08.00-20.00) demonstrated statistical differences

between these and three of the four time slots of peak shedding (03.00-03.59, 04.00-04.59 and 06.00-06.59), suggesting that peak shedding does not occur during the day time.

Over the sampling period, three previously described (Morgan et al., 2017) *Eimeria* spp. were observed, with *Eimeria apteryxi* (present on 14/18 sampling dates) and *Eimeria kiwii* (present on 15/18 sampling dates) most commonly encountered and *Eimeria paraurii* only found on one night in a single sample. As such the results of this research only pertain to *E. kiwii* and *E. apteryxi*.

Results indicate that at least two of the *Eimeria* spp. affecting brown kiwi exhibit circadian variation in oocyst shedding, with a peak output in the early hours of the morning and few oocysts being shed before midnight. This is in contrast with the circadian rhythm reported in most other species of avian coccidia, where the synchronised emergence of oocysts peaks in the late afternoon with few oocysts shed outside of this time (Brawner III & Hill, 1999; Brown et al., 2001; Coelho et al., 2013; Dolnik, 1999a, 1999b; Dolnik et al., 2011; Hudman et al., 2000; Lindström et al., 2009; Lopez et al., 2007; Misof, 2004; Morin-Adeline et al., 2011; P. S. d. Oliveira et al., 2017; Villanúa et al., 2006). This apparent transposition of approximately 12 hours is not surprising considering the kiwis nocturnal life history, where the daylight hours are spent roosting in a burrow and activity generally occurs from around dusk until dawn (Cunningham & Castro, 2011; Heather & Robertson, 2000; Holzapfel et al., 2008; JA McLennan et al., 1987). Although previous research on the oocyst shedding pattern of nocturnal animals is scarce, one previous study investigating two species of *Eimeria* from the nocturnal deer mouse (*Peromyscus maniculatus*) also demonstrated a peak in oocyst shedding during darkness, although one of these *Eimeria* sp. also showed another peak during the day time (Fuller et al., 1995).

Day time data was limited in the present study on kiwi, and it was not possible to determine the exact time of excrement for these samples. However, evidence suggests

that peak shedding does not occur between 08.00 and 20.00. Thus, further research is required to describe any oocyst shedding pattern that may occur during the day time.

2.5.1. Management Implications

The observed circadian periodicity of oocyst shedding has important implications for the management of captive-reared kiwi as estimations of coccidia intensity and prevalence based on oocyst counts without taking the time of excrement into consideration are not reliable (Boughton, 1937c; Brawner III & Hill, 1999; Coelho et al., 2013; Filipiak et al., 2009; Lindström et al., 2009; Lopez et al., 2007; Misof, 2004; Morin-Adeline et al., 2011). Only droppings collected during times of peak oocyst shedding will accurately reflect the true coccidia burden of an individual and samples collected outside of this time may result in either low or false-negative results, even for large coccidia burdens (Coelho et al., 2013; Lopez et al., 2007; Morin-Adeline et al., 2011). The implications of this may include not treating an individual with a significant coccidia burden, with potential for individual or population morbidity, as well as indirect effects such as inappropriately releasing an infected individual thought to be negative on pre-release screening.

2.5.2. Evolution of circadian variation in oocyst shedding

There are currently two hypotheses that attempt to explain the evolutionary forces behind the development of this adaptive trait, neither of which aptly explain findings of the present study in kiwi. The first hypothesis suggests that increased oocyst shedding occurs during times of increased host feeding activity resulting in an increased probability of subsequent ingestion by conspecifics sharing communal feeding areas (Berto et al., 2011; Coelho et al., 2013; Dolnik et al., 2011; Lopez et al., 2007; Martinaud et al., 2009). However, brown kiwi do not have a defined peak in their feeding activity, and rather spend approximately 75% of their active time foraging (Cunningham & Castro, 2011), therefore this theory fails to explain the observed circadian cycle of oocyst shedding in brown kiwi. This hypothesis has already been questioned by Dolnik et al. (2011) who showed the persistence of circadian periodicity in oocyst shedding in snow buntings (*Plectrophenax nivalis nivalis*), despite a lack of

consistent diurnal foraging behavior in the constant daylight of the Arctic. The second hypothesis suggests afternoon shedding allows the vulnerable unsporulated oocysts time to sporulate during the night before being exposed to damaging sunlight the following day (Berto et al., 2011; Coelho et al., 2013; Dolnik et al., 2011; Filipiak et al., 2009; Martinaud et al., 2009). However, it is unlikely oocysts shed by kiwi between 03.00 and 07.00 will have time to sporulate before being exposed to sunlight the next morning. Thus the two current hypotheses regarding the reasons behind the evolution of this adaptive trait appear unable to explain the observed circadian periodicity of oocyst shedding found in brown kiwi. This suggests that either *Eimeria* spp. affecting kiwi have been exposed to differing selection pressures than other avian coccidia species, or that the current hypotheses do not provide a complete explanation for the selection pressures leading to circadian periodicity of oocyst shedding in birds.

Table 4
A summary of the results for dropping sample collection and subsequent oocyst counts for each brown kiwi (*A. mantelli*) sampled at the research site

Kiwi	Number of Sampling Dates	Total number of samples (positive)	Average samples per sampling date	Oocyst count range (opg)	Prevalence (positive nights/ positive samples)	Days Since Toltrazuril Range
1	5	23 (18)	5.5	0-328080	100%/78%	1-9
2	2	15 (12)	7.5	0-11280	100%/80%	5-6
3	4	13 (6)	3.2	0-30160	75%/46%	18-29
4	7	31 (22)	4.6	0-28240	100%/71%	1-5
Total	18	82 (58)	4.5	0-328080	94%/70%	1-29

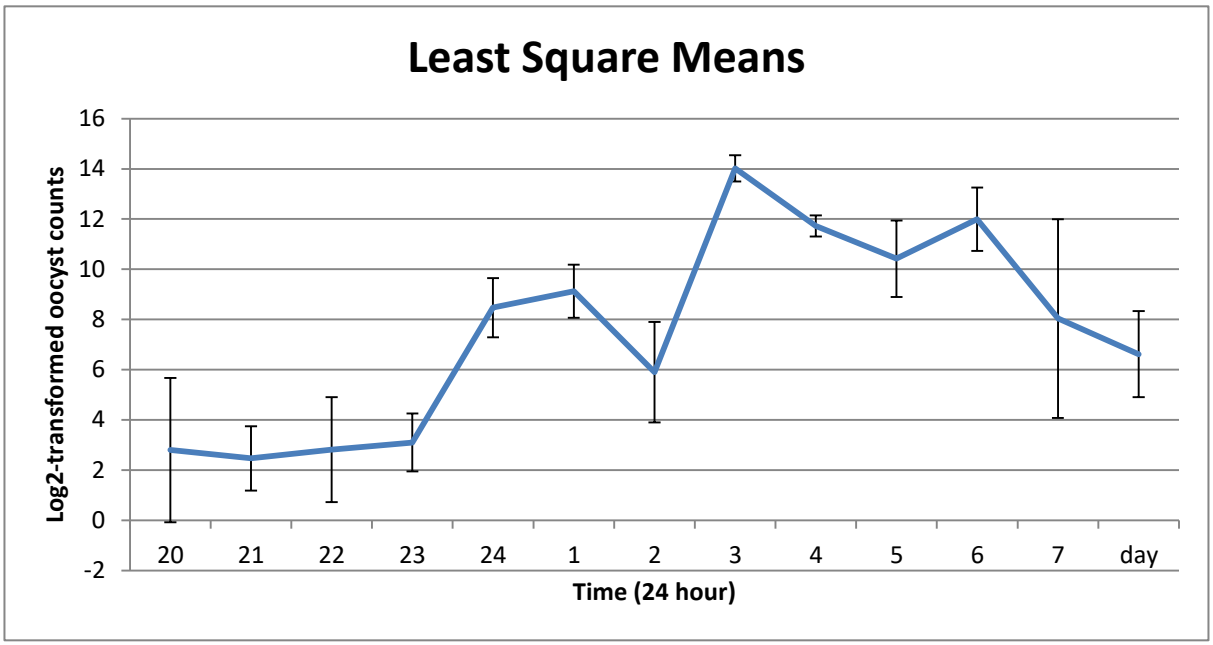

Figure 1 - Least square means of Log2-transformed oocyst counts for each one hour time slot for two species of *Eimeria* affecting brown kiwi at the research site. The bars show standard errors. The day time slot includes all samples collected between 08.00 and 20.00.

2.6. Conclusion

The findings of this research increase our understanding of the biology of two of the *Eimeria* spp. affecting brown kiwi and lead to important implications with regard to the ongoing sustainable management of the kiwi captive breeding programme. Accounting for the circadian variation in oocyst shed allows appreciation of the limitations of current testing methods, as without knowledge of the time of excretion it cannot be distinguished whether a low oocyst count is due to a low burden of *Eimeria* spp., or due to sampling at the wrong time of the day. Further to this, these results may have wider implications applicable to other intensively managed nocturnal species, as well questioning the current theories to explain the evolutionary forces behind the development of this adaptive trait in other avian hosts.

2.7. Acknowledgements

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Chapter Three: The apparent lack of efficacy of toltrazuril against the *Eimeria* species affecting brown kiwi (*Apteryx mantelli*)

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3.1. Abstract

AIM: To assess the efficacy of toltrazuril against the *Eimeria* spp. affecting brown kiwi (*Apteryx mantelli*) at the research site.

METHOD: Dropping samples were collected from brown kiwi at an Operation Nest Egg (ONE) site using video surveillance to establish the time of excrement. Subsequent oocyst counts on droppings from peak-shedding times (0300-0700) were analysed in relation to the days since the most recent toltrazuril administration.

RESULTS: High prevalence and abundance of *Eimeria* spp. oocysts in droppings collected from brown kiwi at the ONE facility were found despite resent toltrazuril administration.

CONCLUSION: These results are unexpected given the reported decrease in oocyst counts for other avian species soon after toltrazuril administration, and suggest that the efficacy of toltrazuril against the *Eimeria* spp. affecting brown kiwi at the research site is deficient. This apparent lack of efficacy appears most likely to be due to the development of resistance.

CLINICAL RELEVANCE: Toltrazuril is widely used at ONE facilities to limit the effects of coccidiosis in captive-reared kiwi, therefore resistance to toltrazuril may directly threaten the sustainability of ONE.

KEY WORDS: *Coccidia, Eimeria, kiwi, Apteryx, toltrazuril, resistance*

3.2. Introduction

In order to reduce the decline of the at-risk brown kiwi (*Apteryx mantelli*) (Robertson et al., 2017), a flightless ratite endemic to New Zealand, a recovery programme known as Operation Nest Egg (ONE) was established in 1995 (Bassett, 2012; Colbourne et al., 2005). During the process of ONE, kiwi eggs or recently hatched chicks are removed from the wild and raised in captivity in order to avoid predation, which is currently the biggest threat to wild kiwi (Bassett, 2012; Colbourne et al., 2005; Holzapfel et al., 2008). Juvenile kiwi are subsequently released back into the wild once a predator-resistant weight of approximately one kilogram is achieved (Bassett, 2012; Colbourne et al., 2005; Holzapfel et al., 2008; JA. McLennan et al., 2004). The ongoing success of ONE is vital for the short term recovery of this species (Bassett, 2012; Colbourne et al., 2005; Holzapfel et al., 2008). Compared to being hatched and raised under natural conditions, ONE invariably leads to a high stocking rate of immunologically naïve kiwi which may result in a higher prevalence of coccidiosis in these birds (Bassett, 2012; Morgan, 2013). Coccidia are one of the most prevalent and important pathogens

affecting kiwi (Bassett, 2012; Doneley, 2006; Morgan, 2013) and has the potential to limit the successful rearing of kiwi through ONE as a result of both clinical and subclinical disease (Morgan et al., 2014). As a consequence, many ONE facilities rely on regular treatment with toltrazuril to control coccidia (Doneley, 2006; Morgan, 2008). Toltrazuril is a symmetrical triazinone and has been shown to be a highly efficacious anticoccidial compound for treating intestinal coccidiosis in other avian species (Chapman, 1987; Claeskens et al., 2007; Sokół et al., 2014; Van Reeth & Vercruysse, 1993; Vertommen et al., 1990). However, recent research at a large ONE facility found a high number of oocysts present in kiwi droppings despite recent oral administration of toltrazuril. The aim of this study is to analyse the effectiveness of toltrazuril to treat the *Eimeria* spp. affecting brown kiwi at the research site.

3.3. Materials and methods

This study was conducted opportunistically during a previous research project on *Eimeria* spp. affecting brown kiwi. The study period ran from the 22nd of February 2017 (sunrise; 0651 sunset; 2004 NZDT) to the 20th of April 2017 (sunrise; 0747 sunset; 1839 NZDT) providing for 16 sampling dates. Four juvenile ONE kiwi were available to be monitored. These birds were housed individually in small outdoor enclosures, measuring approximately 30m^2 and containing grass and small trees, which allowed samples to be clearly identified to a single bird. The time of excrement of these samples was established using video surveillance and only dropping samples that were excreted during peak shedding times (0300-0700) (Taylor, Morgan, Pomroy, McInnes, & Lopez-Villalobos, 2018) are included for this analysis (n = 33). Samples collected during non-shedding times are considered unreliable and are unlikely to accurately reflect coccidia prevalence or abundance (Brown et al., 2001; Coelho et al., 2013; Filipiak et al., 2009; Lopez et al., 2007; Misof, 2004). Oocyst counts were subsequently carried out on each sample using the technique previously described (Taylor et al., 2018). In brief, a known weight of dropping was homogenised with saturated salt solution and oocysts were subsequently counted using the mini-FLOTAC counting chamber (Cringoli et al., 2017). Note that for kiwi dropping samples it is not possible to

separate urate components from intestinal components. Oocysts were quantified and *Eimeria* species identified (Morgan et al., 2017). Where multiple samples from an individual bird are collected on a single date, due to the intermittent nature of oocyst shedding (Taylor et al., 2018), we consider the highest oocyst count to be most representative of the actual oocyst count.

Independent of this study, concurrent management of all juvenile kiwi at this ONE facility included regular monitoring of droppings for the presence of *Eimeria* spp. oocysts. Birds with positive samples were treated with oral toltrazuril at a dose rate of 25mg/kg (Baycox^{TM} coccidiocide for piglets, 50mg/ml, Bayer, Leverkusen, Germany). As a result many of the samples collected during this study were within a short time period after toltrazuril administration. For each sample, the time between the most recent toltrazuril administration and sampling was recorded in days, with the day of treatment being Day 0.

3.4. Results

A total of 33 samples were collected from four birds during the sampling period. Oocyst counts varied from zero to 328,080 oocysts per gram (opg). Sample collection varied from 1-29 days after toltrazuril administration, with 88% (14/16) of sampling dates being within 10 days of toltrazuril administration. The samples from one bird were all more than 19 days after treatment whereas samples from the other three were all within nine days of treatment. *Eimeria* spp. oocysts were identified in 91% (30/33) of individual samples, and on 94% (15/16) of the sampling occasions. Oocyst abundance was very high, with at least one high count (>10,000opg) occurring on 82% of sampling occasions (13/16) (Table 5). Three species of *Eimeria* were observed during this research with *Eimeria apteryxii* and *Eimeria kiwii* most commonly encountered whereas only one sample contained *Eimeria paraurii*. For this reason, subsequent comments on toltrazuril efficacy only pertain to *E. kiwii* and *E. apteryxi*.

3.5. Discussion

The results of this study indicate there is high prevalence and abundance of *E. apteryxii* and *E. kiwii* at the study site despite recent and regular toltrazuril administration. The overall prevalence in these birds is higher than previous reports at other research sites which indicated prevalence between 24-50% (Jakob-Hoff et al., 1999; Morgan et al., 2014). The high prevalence and abundance of *Eimeria* spp. oocysts in the present research is particularly unexpected given the recent administration of toltrazuril and these results raise questions about the current use of toltrazuril for control of this common disease of juvenile kiwi.

Toltrazuril is a highly efficacious coccidiocide used for treating intestinal coccidiosis in many avian species (Chapman, 1987; Mehlhorn et al., 1984; Ramadan et al., 1997; Sokół et al., 2014; Van Reeth & Vercruysse, 1993), however there are no reports of its effectiveness against extra-enteric forms of coccidia. It is efficacious against all intracellular stages (meronts and gamonts) but already formed oocysts do not appear to be susceptible (Harder & Haberkorn, 1989; Mehlhorn et al., 1984; Peek & Landman, 2011). Once preformed oocysts have been excreted, complete elimination or highly significant reductions (88-100%) in the number of oocysts shed are observed within 2-4 days of treatment (Melhorn *et al.* 1984; Chapman 1987; Vertommen *et al.* 1990; Van Reeth and Vercruysse 1993; Ramadan et al. 1997; Peek and Landman 2011; Sokół et al. 2014). Thereafter, low or zero oocyst counts will occur until new infections can establish, depending on the length of the pre-patent period. A further feature of toltrazuril is that it has been shown to have persistent activity in other avian host species, including pigeons and poultry, impairing the development of coccidia for 7-14 days (Van Reeth & Vercruysse, 1993; Vertommen et al., 1990). Thus it would be expected that no oocysts will appear in the droppings until both the period of persistent activity has finished, and then the prepatent period of the particular coccidial species has also passed. Although the prepatent period for the *Eimeria* spp. affecting kiwi is currently unknown, if the persistent activity of toltrazuril and the prepatent period duration is similar to other avian coccidia (e.g. poultry coccidia 3-6

days (McDougald, 2003)), then it would be expected that no oocysts would be observed in the droppings for at least 10 days following treatment.

Although toltrazuril has been used to treat coccidiosis in kiwi for many years, there are no published reports demonstrating its efficacy against *Eimeria* spp. in this host (Morgan, 2008). The dose rate for toltrazuril used at the research site (25mg/kg) is at the upper end of dose rates of between 7-24.5mg/kg reported in a variety of avian species (Sokół et al., 2014; Van Reeth & Vercruysse, 1993). Therefore it is unlikely that results are due to under dosing with toltrazuril.

Although enteric coccidial infections are very common in kiwi (Morgan et al., 2012) extra-intestinal coccidia is reported in kiwi, including renal, hepatic, splenic and pulmonary infections (Alley, 2003, 2004; Morgan, 2013; Morgan et al., 2013). There are few reports of extra-intestinal coccidiosis in other avian host species, although renal coccidiosis is reported in waterfowl (Yabsley, 2009; Yabsley et al., 2002), and disseminated visceral coccidiosis (DVC) has been reported in several species of crane (*Grus*. spp) (Novilla & Carpenter, 2004; Spalding et al., 2009). Several anticoccidial drugs are commonly used for treating renal coccidiosis, including amprolium, salinomycin and zoalene, however there appears to be a paucity of information on how effective these treatments are (Yabsley, 2009). Although there are reports of the successful use of anticoccidials, such as monensisn and trimethoprim-sulphamethoxazole, to treat DVC in cranes (Carpenter et al., 2005; Spalding et al., 2009), some anticoccidial drugs, including amprolium and clazuril, have been shown to lack efficacy against DVC (Carpenter et al., 2005). This may suggest that the control of extra-intestinal coccidia species may be difficult unless an orally administered drug is absorbed in sufficient quantities to produce systemic activity (Carpenter et al., 2005). These previous studies did not include toltrazuril and as such its efficacy against extra-intestinal coccidiosis is unknown. Thus it is possible that toltrazuril fails to effectively treat extra-intestinal forms of coccidia in kiwi, allowing oocyst excretion despite recent toltrazuril administration. However, results from our study in kiwi demonstrate that

toltrazuril appears ineffective against both *E. kiwii* and *E. apteryxii*. Morgan et al (2017) suggests that, although *E. apteryxii* is most likely a renal form of coccidia, *E. kiwii* is most likely to be an intestinal form, and therefore toltrazuril should theoretically be effective against at least this species of coccidia.

It is possible that the population of one or more *Eimeria* spp. affecting brown kiwi at the research site have developed some resistance to toltrazuril due to repeated exposure. The high prevalence and abundance of *Eimeria* spp. oocysts is unexpected when interpreted in conjunction with knowledge on toltrazuril use against at least enteric coccidiosis in other avian host species, and few other plausible explanations for this lack of efficacy are available. Repeated exposure to anticoccidials has led to the development of drug resistance in some isolates of various *Eimeria* spp. (Chapman, 1997; Peek & Landman, 2011; Stephan et al., 1997; Vertommen et al., 1990) and, as with all available anticoccidial treatments, overtreatment with toltrazuril risks the development of drug resistance (Peek & Landman, 2011). The development of resistance to toltrazuril in avian host species has been demonstrated in at least two studies of the *Eimeria* spp. affecting poultry since its release in the late 1980s (Stephan et al., 1997; Vertommen et al., 1990). The treatment protocol for kiwi at the research site commonly results in birds being treated with toltrazuril weekly or fortnightly in response to any positive oocyst output, with the time between treatments occasionally exceeding three weeks and rarely exceeding four weeks. Dropping samples are pooled for each outdoor pen which may contain up to three birds, therefore even if only one kiwi in an enclosure is shedding oocysts, all birds are treated.

The World Health Organization's definition for drug resistance in antimalarial chemotherapy is 'the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject' (W.H.O., 1965) and this can also be applied to anticoccidial compounds (Chapman, 1997; Peek & Landman, 2011). Using this definition, results obtained in the present study suggest that the

populations of *Eimeria* spp. affecting brown kiwi at the research site appear to possess an ability to survive exposure to toltrazuril, and as such it appears probable that some resistance to toltrazuril has developed. It is suggested by Stephan et al. (1997) that anticoccidial resistance under practical conditions is only present when deteriorating weight gain and feed conversion in conjunction with oocyst excretion, intestinal lesions, and mortality give a conclusive overall picture. As no increase in the clinical signs of coccidiosis or a diminishment in the rate of weight gains have been anecdotally reported at the research site, it is probable that this 'practical resistance' has not yet been reached. However, it is also known that oocyst production can correlate poorly with clinical signs and intestinal lesions for some coccidia species (Stephan et al., 1997), making this an unreliable method of surveillance. Further to this, as ONE kiwi are an at-risk wild species (Robertson et al., 2017) being kept in captivity it would not be acceptable to rely on an increase in morbidity or mortality in order to determine resistance.

Unfortunately, no immediate pre-treatment oocyst counts were available to directly compare with post-treatment counts, and further to this, kiwi at the research site are treated regularly based on test results, yet only the most recent toltrazuril treatments were recorded. However, any these earlier treatments with toltrazuril could only result in decreased oocyst counts, it is even more unexpected that high counts were observed. Even though the number of birds studied is small, it is evident that toltrazuril is not having the expected effect on oocyst counts for kiwi at the research site.

The sustainability of ONE relies on coccidiosis control and toltrazuril is currently the only recommended and widely used treatment for *Eimeria* spp. in kiwi (Doneley, 2006; Morgan, 2008). We suggest that the continued reliance on toltrazuril may lead to increased morbidity and mortality at this ONE facility in the future. Further to this, the release of kiwi infected with toltrazuril resistant *Eimeria* spp. into wild populations, or their transfer to other ONE populations, may have subsequent consequences, particularly if treatment should be required in that population in the future.

In order to better understand the efficacy of toltrazuril against *Eimeria* spp. of kiwi at this research site and others, a wider investigation is warranted. If resistance is confirmed, it will be necessary to investigate alternative treatment regimens alongside a broader environmental management strategy to ensure the ongoing viability of ONE.

Table 5
Summary of oocyst count results for samples collected from four brown kiwi (*A. mantelli*) during peak shedding time (0300-0700) at the research site.

Key; EA = *Eimeria apteryxii*, EK = *Eimeria kiwii*

Bird ID	Date of sampling	Days since toltrazuril administration	Number of samples (positive)	Highest count (oocysts/g)	*Eimeria* spp. identified
1	22-23 Feb	4	1 (1)	13120	EA, EK
1	25-26 Feb	1	4 (4)	17600	EA, EK
1	1-2 Mar	5	4 (4)	88720	EA, EK
1	4-5 Mar	8	1 (1)	123440	EA, EK
1	5-6 Mar	9	3 (2)	328080	EA, EK
2	27-28 Feb	5	4 (3)	2160	EK
2	28-1 Mar	6	1 (1)	11280	EK
3	22-23 Mar	19	1 (0)	0	-
3	1-2 Apr	29	2 (2)	30160	EA, EK
4	3-4 Apr	3	2 (2)	23960	EA, EK
4	8-9 Apr	1	2 (2)	21240	EA, EK
4	9-10 Apr	2	3 (3)	18400	EA, EK
4	10-11 Apr	3	1 (1)	18680	EA, EK
4	11-12 Apr	4	2 (2)	28240	EA, EK
4	18-19 Apr	4	1 (1)	7640	EA, EK
4	19-20 Apr	5	1 (1)	12360	EK

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Chapter Four: General Discussion

4.1. Circadian variation in oocyst shedding

Although previous authors suspected circadian variation in oocyst shedding for brown kiwi (Morgan, 2013), this research is the first to confirm and describe this phenomenon. Circadian variation in oocyst shedding is widely reported in other avian hosts, where the synchronised emergence of oocysts generally peaks in the late afternoon with few oocysts shed outside of this time (Brawner III & Hill, 1999; Brown et al., 2001; Coelho et al., 2013; Dolnik, 1999a, 1999b; Dolnik et al., 2011; Hudman et al., 2000; Lindström et al., 2009; Lopez et al., 2007; Misof, 2004; Morin-Adeline et al., 2011; P. S. d. Oliveira et al., 2017; Villanúa et al., 2006). Only one previous report of circadian variation in oocyst shedding for a nocturnal host species was found in the literature (Fuller et al., 1995). This research confirms that at least two species of *Eimeria* affecting kiwi demonstrate a circadian rhythm of oocyst shed, peaking between 3-7am. Outside of this peak shedding, low or negative counts were common, even for kiwi with large *Eimeria* spp. burdens. Given the nocturnal biology of the kiwi, this 12-hour transposition in output compared with diurnal hosts is not surprising. Experimental limitations restricted the collection of day time samples, and although the day time slot (8am-8pm) was significantly different ($p<0.05$) to three of the four time slots of peak shedding, further research is required to determine if, for example, a second peak occurs during the day time, as is the case for the only other nocturnal host species investigated so far (Fuller et al., 1995). Although access to samples during the day was limited in the current research project, further research into daytime oocyst output is warranted.

4.1.1. Management implications for kiwi

Currently, diagnosis of coccidiosis in captive kiwi relies on quantification of oocysts in droppings. Samples are usually collected from enclosures during the day, without information on the time of excrement. Techniques for sample collection vary between

a single sample to pooled samples over several days, depending on the captive institution. The demonstrated circadian variation in oocyst shedding in kiwi means that only droppings collected during times of peak oocyst shedding will likely accurately reflect the true coccidia burden of an individual (Coelho et al., 2013; Morin-Adeline et al., 2011). Coccidia detection and quantification techniques have been shown to be inaccurate in other species that undergo this circadian variation in oocyst shedding if time is not accounted for (Filipiak et al., 2009; Lindström et al., 2009; Lopez et al., 2007; Morin-Adeline et al., 2011). Therefore, estimations of *Eimeria* spp. burden intensity and prevalence for brown kiwi based on oocyst counts that do not take the time of excrement into consideration may be unreliable and cannot be accurately interpreted (Boughton, 1937c; Brawner III & Hill, 1999; Coelho et al., 2013; Filipiak et al., 2009; Lopez et al., 2007; Misof, 2004; Morin-Adeline et al., 2011). Thus current testing regimens are at high risk of obtaining false low or false negative results if samples collected are from outside this 3-7am period. The findings in this study indicate that oocyst counts do not just decrease a little during the day as even high overnight counts may be zero if droppings are collected during daylight hours. Implications of a false negative may include not treating an individual with a significant coccidia burden, increasing the risk of morbidity or mortality. As coccidiosis may have significant impacts on growth and weight gain (Dalloul & Lillehoj, 2006), non-treatment of infected birds may result in kiwi taking longer to reach their target release. This is an important consideration for ONE institutes as there are high financial costs, staff time and space limitations associated with kiwi requiring longer at an ONE institute before release. Other impacts include exacerbation of environmental contamination with oocysts; release of an infected individual which may result in appearance of disease due to the stress of translocation; release of high burdens of oocysts into the release site; and spread of novel coccidia to a potentially naïve wild population.

4.1.2. The evolution of circadian variation in oocyst shedding

Currently two hypotheses are proposed to explain the circadian variation in coccidia oocyst shedding for avian host species (Berto et al., 2011; Dolnik, 1999a; Martinaud et

al., 2009; Misof, 2004). The first hypothesis suggests that oocyst release corresponds to a peak of host feeding activity (Berto et al., 2011; Lopez et al., 2007; Martinaud et al., 2009). This leads to many susceptible individuals being present in the communal feeding area where maximum oocyst excrement occurs, resulting in a higher probability of subsequent oocyst ingestion (Lopez et al., 2007; Martinaud et al., 2009). All previous studies on the circadian variation in oocyst shedding for avian hosts have been carried out on diurnal species and maximal oocyst shedding has coincided with a peak in feeding activity during the late afternoon (Berto et al., 2011; Coelho et al., 2013; Dolnik, 1999a, 1999b; Lopez et al., 2007). In contrast, brown kiwi are nocturnal and generally active only at night (Castro & Morris, 2011; JA McLennan et al., 1987), during which they spend approximately 75% of their time foraging (Cunningham & Castro, 2011) with no defined peak in feeding activity. Without a peak in feeding activity, there appears no apparent reason why oocysts shed by kiwi between 3am and 7am would have an increased chance of subsequent ingestion by conspecifics. Similarly, Dolnik (2011) showed the persistence of circadian periodicity in oocyst shedding by snow buntings in the Arctic where constant daylight results in a lack of consistent diurnal foraging behavior patterns. Further to this, many passerine species also have a period of high activity in the morning which does not have a corresponding increase in oocyst shedding (Berto et al., 2011; Coelho et al., 2013).

The second hypothesis to explain the circadian periodicity of oocyst shedding suggests that the observed afternoon shedding allows vulnerable unsporulated oocysts time to sporulate over night before being exposed to damaging sunlight the following day (Coelho et al., 2013; Filipiak et al., 2009; Martinaud et al., 2009). Before sporulation, oocysts are vulnerable to desiccation (Berto et al., 2011; Dolnik et al., 2011; Martinaud et al., 2009; McQuistion, 2000) and if oocysts were shed constantly throughout the day many would die from desiccation due to sunlight exposure (Coelho et al., 2013; Martinaud et al., 2009). Martinaud et al. (2009) showed that exposure to sunshine reduced oocyst viability dramatically, with one hour of exposure reducing oocyst viability by approximately 50% and after four hours few oocysts remained viable. Thus

afternoon shedding allows the sporulation of oocysts overnight making them more resistant to sunlight and resulting in higher survival rates (Dolnik et al., 2011; Martinaud et al., 2009). Sporulation is thought to take 18 hours to four days for *Isospora* spp. affecting passerines (Dolnik et al., 2011), and 12 to 30 hours for *Eimeria* spp. affecting chickens (McDougald, 2003); these timeframes are probably sufficient for the majority of oocysts to sporulate overnight before being exposed to sunlight the following day. The sporulation time for the *Eimeria* spp. affecting brown kiwi are currently unknown and further research is required in this area. However, the time between the observed peak of oocyst shedding in kiwi and sunrise the following day is approximately 4-8 hours, which is unlikely to be adequate to allow sufficient time for sporulation. In addition, being predominantly bush dwellers (Jamieson, Castro, Jensen, Morrison, & Durrant, 2016), kiwi are likely to excrete the majority of their droppings in areas of full shade, and these oocysts are unlikely to ever be exposed to direct sunlight. Therefore, it is unlikely that sunlight avoidance is the driving factor for the evolution of this adaptation for the *Eimeria* spp. affecting brown kiwi.

Neither current hypothesis appears to explain the observed circadian periodicity of oocyst shedding found in brown kiwi in the present study; being shed between 3am and 7am is unlikely to increase ingestion by conspecifics due to increased feeding activity or decrease desiccation due to allowing sporulation prior to exposure to sunlight. Instead, results of this research suggest that either kiwi coccidia have been exposed to different selection pressures to previously-described coccidia species, or the previous hypotheses as to selection pressures for development of this trait are incorrect for at least some species of coccidia. As there is very little published literature on this trait in nocturnal avian species, determination of oocyst shed patterns in other nocturnal hosts may assist in clarification of the selection pressure in nocturnal species.

This present research found that the oocyst shedding pattern of brown kiwi, a nocturnal species, is transposed by approximately 12 hours compared to that of the previously investigated diurnal host species (Dolnik, 1999b; Misof, 2004; Morin-Adeline

et al., 2011). This concurs with Fuller et al. (1995), who demonstrated that two *Eimeria* spp. affecting a nocturnal species of mouse show a peak in shedding during the dark. This suggests that oocyst shedding corresponds to the time the host is awake, rather than daylight hours. This is supported by Boughton (1933), who demonstrated that reversing the light regime, which in turn reversed the awake-sleep cycle, resulted in the exact opposite oocyst shedding pattern in sparrows. Thus it is possible that a factor directly related to the hosts awake sleep cycle may be behind the evolution of circadian variation in oocyst shedding for coccidia species affecting avian hosts. Ultimately the exact selection pressures behind the development of this circadian variation in oocyst shedding of coccidia species affecting avian hosts, including *Eimeria* spp. affecting brown kiwi, remain a mystery (Dolnik et al., 2011; Martinaud et al., 2009).

4.1.3. Oocyst shedding triggers

Currently there are several proposed theories regarding the possible underlying mechanisms that trigger oocyst shedding (Lopez et al., 2007; Misof, 2004). The adaptive value of circadian rhythms for an organism is in the advantage of synchronising its physiology and behaviour with the periodically changing environment and, for parasites, these environmental changes are experienced via their hosts (Dolnik et al., 2011). Thus the variation in oocyst shedding must be controlled by an aspect of the host's physiology (Coelho et al., 2013; Lopez et al., 2007). Further to this, as oocyst shedding undergoes circadian variation it is likely that the mechanism responsible for triggering oocyst shedding also undergoes a circadian cycle.

One suggested mechanism is the circadian variation in photoperiod and melatonin (Dolnik et al., 2011). However, results of the present research show that brown kiwi undergo shedding at a time that is transposed by approximately 12 hours compared to that of other avian hosts, despite the fact that nocturnal and diurnal animals both experience peak melatonin levels at the same time in response to darkness (Challet, 2007; Grant, 2012; Saper et al., 2005). This lack of correlation between oocyst output and melatonin levels is consistent with findings in snow buntings undergoing constant

daylight in the Arctic, where melatonin levels are expected to be constant, with circadian periodicity of oocyst shedding persisting for these birds (Dolnik et al., 2011). Further to this, artificially altering the melatonin levels in birds does not appear to synchronise oocyst output rhythms for *Isospora* spp. ((Brandlmeier) as cited by Dolnik at al., 2011).

An increase in host activity, such as that observed during peak feeding times, has been implicated as another trigger for oocyst shedding (Dolnik et al., 2011; Lopez et al., 2007). The present research in kiwi refutes this because, despite constant activity of kiwi throughout the night (Cunningham & Castro, 2011), there is still a distinct period of peak oocyst output. Further to this, Dolnik at al. (2011) found the persistence of circadian variation in oocyst shedding despite a lack of consistent host foraging activity in snow buntings, and passerine species generally show an increase in activity in the morning with no corresponding increase in oocyst shedding (Berto et al., 2011; Coelho et al., 2013; McQuistion, 2000).

The host's sleep-wake cycle has been suggested as an alternative trigger (Boughton, 1988; Misof, 2004). The present research concurs with this idea finding that the kiwi, a nocturnal species, exhibits approximately the opposite circadian pattern of oocyst shedding. Investigating the oocyst shedding pattern of captive display kiwi that are kept in dark houses, where their sleep-wake cycle is reversed, may provide insight to this theory. Further research on other nocturnal species' oocyst shedding patterns may also be useful.

4.2. Species of *Eimeria* affecting brown kiwi

In order to differentiate *Eimeria* spp. using morphological techniques, oocyst length and width were measured, as described by Morgan et al. (2017). Although unsporulated oocysts were measured in the present research, rather than sporulated oocysts as reported by Morgan et al. (2017), pre and post sporulation measurements are not expected to change for oocysts (Duszynski, 1971). In addition, there is limited overlap in oocyst size and shape between *Eimeria* species affecting brown kiwi

(Morgan et al., 2017), adding further weight to this as an acceptable method of differentiating these species.

Morgan et al. (2017) previously described four species of *Eimeria* currently known to affect brown kiwi from two research sites, with *E. apteryxii* and *E. kiwii* the predominant species. Birds frequently show infection with more than one species. Although the present research was undertaken at a different site, these were also the predominant species identified, with only one sample containing *E. paraurii* and none showing *E. mantellii*. Many samples showed mixed infections, although samples showed variation in *Eimeria* spp. incidence and proportional representation between subsequent samplings.

As the research site has housed kiwi from three of the four taxa of brown kiwi as well as two other species of kiwi, it is likely that the research site has been exposed to a higher number of species of *Eimeria* than these two. Potential explanations for our results may include differing fecundity of *Eimeria* spp.; varying susceptibility of *Eimeria* spp. to toltrazuril; and seasonal variations in coccidia prevalence.

Although the two species of *Eimeria* often occurred in different proportions, both species appeared to exhibit the same general pattern of oocyst shedding when mixed infections were observed, agreeing with previous work in chickens (P. P. Levine, 1942). However, it is possible that *E. apteryxii* and *E. kiwii* have slightly varying oocyst shedding patterns and further research is required to determine their individual oocyst shedding patterns. Further to this, research into the oocyst shedding cycle of both *E. mantellii* and *E. paraurii* is required.

4.2.1. Management implications

Coccidia is a natural parasite of free living kiwi often occurring without clinical disease being evident (Bassett, 2012; Jakob-Hoff et al., 1999; Morgan et al., 2014), thus its presence alone does not infer a problem. Instead it is the risk factors present in ONE institutes – high stocking rate of young, immunologically naïve kiwi – that are the reason coccidia becomes a problem (Jakob-Hoff et al., 1999; Morgan et al., 2012;

Morgan et al., 2013; Morgan et al., 2014). As two major risk factors for coccidia, young age (Jakob-Hoff et al., 1999; Morgan et al., 2013; Morgan et al., 2014) and increased stocking rate (Morgan et al., 2012; Morgan et al., 2013), seldom occur in conjunction in the wild it is unlikely that releasing ONE kiwi that are infected with coccidia is a direct threat to individual wild kiwi. However, an important consideration is the potential for exposing a wild kiwi population to a species of *Eimeria* that they have not previously encountered via the release of an infected ONE kiwi (Morgan et al., 2013; Morgan et al., 2014). Host immunity to *Eimeria* spp. develops via multiple rounds of exposure and recovery from specific *Eimeria* spp. (Shirley & Lillehoj, 2012), and is not necessarily a direct effect of age (Yun et al., 2000). Further to this, immunity to one *Eimeria* species may not infer cross-protection to another *Eimeria* spp. (Joyner & Long, 1974; Long & Joyner, 1984), suggesting that exposure to one of the four described *Eimeria* spp. affecting kiwi may not provide cross-immunity to the other *Eimeria* spp.. Therefore wild kiwi will likely lack immunity to any novel species of *Eimeria* they have not previously been exposed to. This current research, in conjunction with previous research (Morgan et al., 2017), suggests that differences in the presence of different *Eimeria* spp. occur between captive populations. This may also mean that different wild kiwi populations have different *Eimeria* spp. present. Thus the release of ONE kiwi infected with a particular *Eimeria* spp. may risk introducing a novel species of *Eimeria* to a naïve population of wild kiwi (Morgan et al., 2013; Morgan et al., 2014), and the possibility of a disease outbreak exists due to the naïve wild populations lack of immunity. Exposure to a novel species of *Eimeria* is also a risk for newly released ONE kiwi. If an individual is released into a wild population where an *Eimeria* spp. exists that the transferring bird has not been exposed to during ONE, they may have no developed immunity to this *Eimeria* species and may be susceptible to illness. Any illness caused by this exposure to a novel species may be further compounded by the stress associated with release, and as a result they may face reduced post-release survival. This has the potential to affect many released birds and may drastically affect the

success of an ONE programme. Further research on the incidence of *Eimeria* spp. within captive and wild kiwi populations is required to quantify these risks.

Coccidian species affecting other avian species are generally host specific or have a narrow host range (Berto et al., 2011; Dolnik, 1999c; Joyner & Long, 1974; Long & Joyner, 1984; Marquardt, 1981; Schoener et al., 2013). However, previous studies have shown that the host specificity of coccidia species varies considerably (Berto et al., 2010; Marquardt, 1981; Norton, 1967; Tung et al., 2007). ONE is currently carried out for all five species of kiwi, all of which have been shown to be susceptible to coccidia (Morgan et al., 2013; Morgan et al., 2014), however it is currently unknown whether each of the five species of kiwi have their own unique *Eimeria* spp. (Morgan et al., 2012; Morgan et al., 2013). The five species of kiwi are from the same taxonomic family and genera (Burbidge et al., 2003; Holzapfel et al., 2008), meaning it is possible that they may be susceptible to the same species of *Eimeria*. However, it is currently unknown how host specific the *Eimeria* spp. affecting brown kiwi are, and whether any of the four described species of *Eimeria* affecting brown kiwi can infect additional species of kiwi (Morgan, 2013; Morgan et al., 2013). Further research is required to determine the interaction, if any, between different kiwi species and *Eimeria* spp.

4.3. Toltrazuril

Although toltrazuril anecdotally appears to be effective in the treatment and control of coccidiosis in kiwi, there have been no robust investigations into its efficacy in kiwi. In other host species, toltrazuril has been shown to be highly effective against all intracellular stages of intestinal coccidia. The only commercially available product in New Zealand (Baycox®) has only been formulated for use against intestinal coccidial species in cattle, poultry and pigs. To our knowledge, there is no published information on the effectiveness of toltrazuril to control visceral forms of coccidiosis in any host species.

Toltrazuril is currently the only anti-coccidial medication commonly used in kiwi, and in at least some management systems, it is used at a very high frequency in young birds.

For example, at the research site for this study, ONE kiwi were usually treated every 1-2 weeks with toltrazuril on account of stool samples which were positive for coccidia. This high frequency of administration poses a high risk for the development of resistance to toltrazuril, as has been demonstrated in other host species.

The current research suggests that toltrazuril may be ineffective against at least some species of coccidia in brown kiwi at one site. Possible reasons for this may include inadequate uptake and metabolism of the drug in kiwi; an inappropriate dose rate; ineffectiveness of toltrazuril against extra-enteric forms; and/or the development of resistance. Because these findings were opportunistic and not one of our research questions, there are limitations to our data, in particular the lack of pre-treatment sampling data. Further research, which includes oocyst counts before treatment with toltrazuril, is required, along with a higher number of kiwi in the data set. Unfortunately, due to the endangered status of kiwi, standard mechanisms to ascertain the effectiveness of toltrazuril, such as inoculation and euthanasia to assess pathological changes due to coccidia, are not warranted. Other research directions to assess the inherent effectiveness of toltrazuril in kiwi include a pharmacokinetic study of this drug in kiwi.

As this study was only performed on brown kiwi at one study site, with two predominant species of *Eimeria*, further research is required to assess the efficacy of toltrazuril in other ONE sites, including other *Eimeria* spp. It may also prove useful to investigate this efficacy in populations that have not previously been exposed to toltrazuril, such as wild populations. The future development of diagnostic molecular tools may replace current morphological techniques to assist in acquisition of more robust data, in particular molecular identification of *Eimeria* spp. and potentially utilization of real time PCR for oocyst quantification.

Conclusion

Coccidiosis is one of the most prevalent and limiting diseases influencing the successful rearing of ONE kiwi (Doneley, 2006; Morgan et al., 2012) and, should control measures fail, it may threaten the ongoing sustainability of this vital recovery programme. Continuing to improve our understanding of host-parasite interactions is vital to enable effective disease management in order to reduce the detrimental impact of coccidiosis on ONE and ensure the ongoing success and sustainability of this important recovery programme. The findings of this research increase our understanding of the biology of the *Eimeria* spp. affecting brown kiwi and have important implications regarding the captive management of kiwi. By describing the circadian variation in oocyst shedding for two *Eimeria* spp. affecting brown kiwi the limitations of current testing methods can be appreciated, highlighting that accurate interpretation of oocyst counts requires knowledge of the time of dropping excrement. Failure to incorporate the time of excrement into the interpretation of oocyst counts from kiwi results in inaccurate conclusions that are likely to lead to poor management decisions. Without knowledge of the time of excrement of a sample it cannot be determined whether a low oocyst count is due to a low *Eimeria* spp. burden or due to sampling an individual with a large *Eimeria* spp. burden during non-shedding times, and thus false low and false negative results are inevitable. Practically applicable solutions to this are currently difficult to find. It is not possible, from a cost and time perspective, to monitor all ONE kiwi with cameras to determine the time of excrement as was done in this research. Pooling samples provides some insurance against the variation in counts between samples. However, during the current research there were several occasions where pooling of samples was inadequate to safeguard against false negative results.

The present research, in conjunction with earlier findings, suggests that the presence of the various *Eimeria* spp. affecting brown kiwi differs between kiwi populations. This may have important implications regarding the appropriate management of the release of ONE kiwi from captive facilities. This research also questions the

effectiveness of toltrazuril to treat coccidiosis in kiwi and further research is urgently required to confirm this lack of efficacy and determine the reasons behind it.

The results of the present research also have wider implications than kiwi management, with the described circadian variation in oocyst shedding by brown kiwi unable to be explained by current theories proposing the evolutionary forces behind the development of this trait. When interpreted in conjunction with earlier research, the results of this present research may provide important inferences on potential triggering mechanisms for oocyst shedding. Further to this, there is a dearth of information on oocyst shedding patterns for nocturnal species and as such these results may have wider implications applicable to other intensively managed nocturnal species.

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Appendices

Appendix 1 Standard Operating Procedure
Sample Collection Method

1. Purpose

To collect droppings samples with a known time of excrement.

2. Equipment and Materials

- Motion triggered cameras capable of video recording in darkness
- Elastic cords
- Sample pottles
- Pen
- Gloves
- Lettered/Numbered plastic pens

3. Safety Considerations

Kiwi droppings may contain zoonotic pathogens and gloves should be worn for handling.
All relevant health and safety protocols for field work should be carried out as detailed in the health and safety plan at the research site.

4. Procedure

A. Camera Placement

Cameras should be set up prior to 5pm to ensure they are in place for filming during darkness. Only kiwi kept alone in pens may be utilised for this method.

1. Locate the kiwi within the pen and block the entrance to its sleeping burrow to remove the possibility of the bird leaving its burrow and being walked on during set up
2. Walk through the entire pen and remove all kiwi droppings to avoid the risk of collecting old samples. Note any areas where multiple droppings occur.
3. Select seven areas suitable for filming as well as sample collection. These should be areas where the camera will be triggered and capture unobstructed video

and the droppings will be intact. Grass areas or areas of thick vegetation should be avoided. Target areas where multiple droppings were found.

4. Using plastic-tipped bungee cords or elastic rope tie the cameras to trees in such a way that they will video the selected areas. Use sticks from within the pens to pack out between the camera and the tree to achieve the correct angle. No metal is to be brought in to the pens.
5. Set all cameras to 60 second videoing with a 1 second re trigger.

B. Sample Collection

Sample collection should occur the morning following videoing.

1. Locate the kiwi within the pen and block the entrance to its sleeping burrow to remove the possibility of the bird leaving its burrow and being walked on during sample collection
2. Search the pen in a systematic way to ensure no samples are missed. Place each sample in a collection pottle and mark it with a letter.
3. Use the plastic peg with the corresponding letter to mark the exact place of collection, ensuring the letter on the peg is facing the camera.
4. Trigger the camera to begin filming by waving in front of it several times.

C. Analysis

Review the video footage from each camera. Only include samples where complete confidence in the time of excrement is achieved. A pictorial example of sample excrement and subsequent peg location is provided below for sample A – 26/2/17 at 4.20am.

1. Each time a dropping is observed place a finger on the screen in the exact location it lands.
2. Move to the last video segment containing the lettered plastic pegs and assign the observed dropping sample to its correct letter.
3. Return to the original video segment showing the dropping occurring and record the exact time for that lettered sample (eg sample F = 9.43pm). Save that file as the sample letter and time.


0:00:24
0:00:06
02/26/2017 04:20:31


0:00:21
0:00:09
02/26/2017 08:40:38

Appendix 2 Standard Operating Procedure
Oocysts Counting Method

1. Purpose;

To quantify the number of oocysts per gram of dropping for each sample.

2. Equipment and Materials

- Scales
- Fine mesh sieve (tea strainer is adequate)
- Stainless steel bowl with approximately 10cm diameter
- Teaspoon
- Pipette
- Measuring beaker
- Saturated sodium chloride solution (specific gravity of 1.2)
- Mini-FLOTAC counting chamber
- Stopwatch
- Light microscope
- Eyepiece micrometer

3. Safety Considerations

No specific safety requirements

4. Procedure

1. Using a sieve over a bowl mix 1 gram of droppings with 19mls of saturated sodium chloride solution (specific gravity 1.2) – this gives a dilution ratio of 1:20. If 1 gram of droppings is not available use the maximum amount available and alter the amount of saturated salt solution to ensure a 1:20 dilution factor is achieved.
2. Homogenise the faeces with the saturated salt solution using a spoon and sieve for 30 seconds.
3. Using a pipette fill the mini-FLOTAC flotation chambers (2x 1ml chambers) via the filling holes, holding the mini-FLOTAC device on a slight angle to avoid air bubbles and facilitate filling until a meniscus is formed.
4. Place the mini-FLOTAC on a flat level surface and leave to stand for 10 minutes.
5. After 10 minutes turn the reading disc clockwise 90 degrees.
6. Place the mini-FLOTAC on the microscope adaptor and view through the microscope at 20x magnification. Starting at the top right corner scan each line

and count the oocysts -at 20x magnification it is necessary to go up one side and down the other side of each line. Measure the length and width of the first 25 oocysts encountered and assign each to a species using oocyst length and width measurements from Morgan et al. (2017). Count all oocysts in the entire chamber including the outside rows with curved corners.

7. If a count of <20 is made on the first line count every line including the outside curved-cornered lines. The overall count should be adjusted by a multiplication factor of 10 (due to examining 2mls of a 1:20 diluted solution). If a count of 20-40 oocysts is made in the first line then only count alternating lines and use a multiplication factor of 20x on the total count. If a count of >40 is made then only count half of each alternating line in 2 opposite quarters of each chamber and use a multiplication factor of 40x on the total count. See the figure below for a pictorial explanation of these options.
8. Multiply the total oocyst count by the percentage of each species of coccidia found to determine the relative abundance of each species of coccidia in the dropping sample.

Alternate rows

Alternate rows and alternate quadrants

Use the left option if the first row count is 41-80 oocysts and the right option if the first row count is >80 oocysts.

Appendix 3 Standardised result recording form

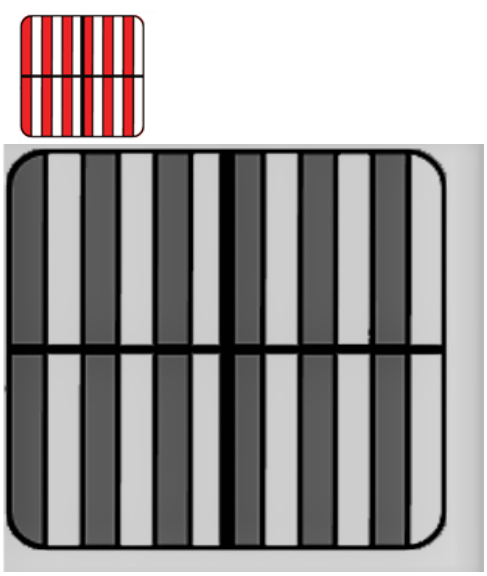
Sample ID; Date sample taken;

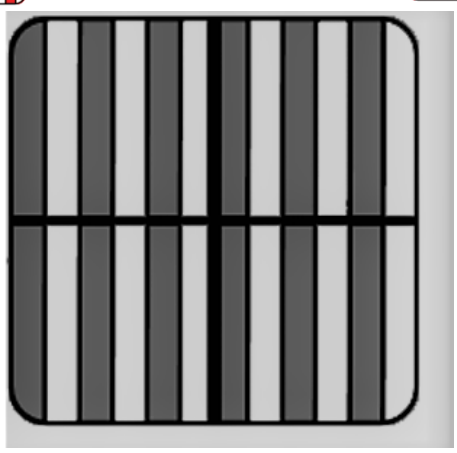
Kiwi; Baycox;

Weight of faces (g); ml saturated salt solution; = 1:20 dilution

Count Technique Used (circle)

Low (MF=10x) Moderate (MF = 20x) High (MF = 40x)





Total G1 = _____ Total G2 =_____

Total both grids; _____ Multiplication Factor = _____

Total Count = _____

Species	Number	%	Total Count	Extrapolated Total
E. apteryxi				
E. kiwii				
E. mantelli				
E. paraurii				
Other				
Total				

Appendix 4 Results of dropping oocyst counts, species presence, exact time of excrement and days since toltrazuril administration for each sampling date for individual kiwi.

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
22/23 February	Moehau (1)	18/2/17	4+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
8:40	0	0	0	0	0
23:43	60	60	0	0	0
5:23	13120	10496	2624	0	0
Day	8880	6038	2842	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
25/26 February	Moehau (1)	24/2/17	1+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
3:25	17600	12672	4928	0	0
4:20	5960	2145	3814	0	0
4:32	13920	8908	5011	0	0
5:29	7460	3879	3580	0	0
Day	5920	4262	1657	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
27/28 Feb	Fissure (2)	22/2/17	5+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
20:26	1760	0	1760	0	0
0:33	30	0	30	0	0
1:00	160	88	72	0	0
1:10	1140	0	1140	0	0
1:44	190	0	190	0	0
4:55	2160	0	2160	0	0
5:30	330	0	330	0	0
5:51	1900	0	1900	0	0
6:42	0	0	0	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
28/1 Feb/Mar	Fissure (2)	22/2/17	6+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
21:23	0	0	0	0	0
21:56	380	0	380	0	0
23:09	0	0	0	0	0
1:12	30	0	30	0	0
1:22	320	0	320	0	0
3:28	11280	0	11280	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
1-2 March	Moehau (1)	24/2/17	5+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
21:24	50	50	0	0	0
21:51	0	0	0	0	0
4:32	88720	17744	70976	0	0
5:45	12760	4083	8676	0	0
5:59	15480	4953	10526	0	0
6:09	24200	1936	22264	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
4-5 March	Moehau (1)	24/2/17	8+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
22:16	180	0	180	0	0
23:06	0	0	0	0	0
4:07	123440	14812	108627	0	0
Day	3890	2256	1867	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
5-6 March	Moehau (1)	24/2/17	9+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
21:12	0	0	0	0	0
5:10	328080	104985	223094	0	0
5:15	0	0	0	0	0
6:44	17440	7673	9766	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
21-22 March	Kenny (3)	3/3/2017	18+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
23.07	0	0	0	0	0
1.59	0	0	0	0	0
Day	0	0	0	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
22-23 March	Kenny (3)	3/3/2017	19+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
0.06	490	490	0	0	0
5.05	0	0	0	0	0
Day	0	0	0	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
27-28 March	Kenny (3)	3/3/2017	24+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
22.19	0	0	0	0	0
23.3	0	0	0	0	0
Day	820	426	394	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
1-2 April	Kenny (3)	3/3/2017	29+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
1.47	1530	184	1346	0	0
3.46	30160	25334	4826	0	0
5.53	14640	11712	2928	0	0
Day	720	576	144	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
3-4 April	Paopao (4)	31st March	3+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
4.25am	4100	3116	984	0	0
4.51am	23960	2875	21085	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Toltrazuril**		
8-9 April	Paopao (4)	7th April	1+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
21.41	230	0	230	0	0
21.58	0	0	0	0	0
23.32	110	0	110	0	0
23.47	600	0	600	0	0
1.23	560	0	560	0	0
6.01	21240	0	21240	0	0
6.37	9160	733	8427	0	0
Day	0	0	0	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Baycox**		
9-10th April	Paopao (4)	7th April	2+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
20.39	0	0	0	0	0
22.32	0	0	0	0	0
4.09	6400	256	6144	0	0
5	18400	1472	16928	0	0
6.23	3360	403	2957	0	0
Day	70	40	30	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Baycox**		
10-11th April	Paopao (4)	7th April	3+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
21.18	0	0	0	0	0
21.38	0	0	0	0	0
6.03	18680	2509	13171	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Baycox**		
11-12th April	Paopao (4)	7th April	4+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
23.22	0	0	0	0	0
0.31	840	0	840	0	0
1.14	8680	347	8333	0	0
3.02	28240	1130	27110	0	0
3.29	15640	626	15014	0	0
7.42	0	0	0	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Baycox**		
18-19th April	Paopao (4)	14th April	4+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
5.12	7640	3056	4584	0	0

Date	**Kiwi**	**Last Dosed**	**Days since Baycox**		
19-20 April	Paopao (4)	14th April	5+		
Time (24h)	**Total Number of Oocysts**	***E. apteryxii***	***E. kiwii***	***E. paraurii***	***E. mantellii***
19.59	0	0	0	0	0
22.5	280	0	280	0	0
6.22	12360	0	12360	0	0
7.01	3940	0	3940	0	0
7.28	1860	0	1860	0	0

Appendix 5 Summary of all oocyst count results, grouped by hour, in relation to time of excrement, date and days since toltrazuril administration

Time of Excrement	22-23 Feb 2017	25-26 Feb 2017	27-28 Feb 2017	28-1 Mar 2017	1-2 Mar 2017	4-5 Mar 2017	5-6 Mar 2017	21-22 Mar 2017	22-23 Mar 2017	27-28 Mar 2017	1-2 Apr 2017	3-4 Apr 2017	8-9 Apr 2017	9-10 Apr 2017	10-11 Apr 2017	11-12 Apr 2017	18-19 Apr 2017	19-20 Apr 2017
20:00																		0
20:30	0		1760											0				
21:00							0								0			
21:30				0	50								230		0			
22:00				380	0								0					
22:30						180				0				0				
23:00				0		0		0										280
23:30	60									0			110			0		
0:00									490				600					
0:30			30													840		
1:00			160	30												8680		
1:30			1140	320									560					
2:00			190					0			1530							
2:30																		
3:00																28240		
3:30		17600		11280												15640		
4:00		5960				123440					30160			6400				
4:30		13920			88720							4100						
5:00			2160				328080		0			23960		18400			7640	
5:30	13120	7460	330		12760		0											
6:00			1900		15480						14640		21240		18680			
6:30			0		24200		17440						9160	3360				12360
7:00																		3940
7:30																0		1860
8:00																		
Day	8880	5920				3890		0	0	820	720		0	70				
Days since Toltrazuril	4	1	5	6	5	8	9	18	19	24	29	3	1	2	3	4	4	5