Cryptosporidium spp. in wild and captive Australian flying foxes (genus: *Pteropus*)

Sabine Eva Schiller, Bachelor of Philosophy

Macquarie University, North Ryde, NSW, 2109, Australia

Department of Biological Sciences

Tel.: 61 2 9850 9259; Fax: 61 2 9850 8245

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Abstract

Spillover of zoonotic pathogens from wildlife to humans is a primary threat to global health, but the potential impacts of reverse pathogen transmission (zooanthroponosis) is still largely unexplored. Increasing establishment of wildlife species in regional and urban Australia potentially increases risk of human-borne pathogen spillover at the human/wildlife interface. To explore this issue, the occurrence of the protozoan parasite *Cryptosporidium* was investigated in urbanised flying fox populations.

Cryptosporidium infects a wide range of vertebrates, with species varying in host specificity. In humans, *C. hominis* and *C. parvum* are responsible for the majority of infections. PCR screening of faecal samples (*n* = 281) from seven wild and two captive flying fox populations identified the presence of *Cryptosporidium* in 3.2% of samples, with a prevalence of 1.7% in wild versus 6.3% in captive individuals ($\chi^2 = 3.708$, DF = 1, p= 0.054). Using multilocus sequencing (18s rRNA, actin and *gp60*) *C. hominis* was identified in captive animals (*n* = 2) and four novel *Cryptosporidium* genotypes in wild and captive animals (*n* = 7). This is the first study to report the presence of *Cryptosporidium* spp. in Australian flying foxes and findings indicate zooanthroponotic transmission of *Cryptosporidium* from humans to flying foxes.

This thesis is written in the form of a journal article from International Journal of Parasitology: Parasites and Wildlife, including an extended introduction/literature review (section1)

Declaration

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Dr. Koa Webster

Jennefer Maclean, Tolga Bat Hospital (Sample collection)

Kerryn Parry-Jones, Wambina Flying Fox Sanctuary (Sample collection)

Tim Pearson, Macquarie University (Sample collection)

I hereby declare that the work in this thesis entitled "*Cryptosporidium* spp. in wild and captive Australian flying foxes (genus: *Pteropus*)" is an original piece of research conducted as part of a nine month Masters of Research project. It has not previously been submitted for a degree to any university other than Macquarie University.

Any assistance that I have received during this project has been appropriately acknowledged along with all relevant information sources and literature in this thesis.

Funding for this project was provided by the Ian Potter Foundation and Macquarie University. I hereby declare that I am not aware of any conflict of interest in relation to this project.

All other research described in this report is my own original work.

Sabine Eva Schiller (Student ID: 41967062)

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Abbreviations

Base pair	bp
Deoxyribonucleic acid	DNA
Emerging infectious diseases	EID
Flying foxes	FF
Flying fox samples as indicated by number	FF38, FF43 etc.
New South Wales	NSW
Polymerase chain reaction	PCR
Restriction fragment length polymorphism	RFLP
Queensland	QLD

Keywords: *Cryptosporidium*, Zooanthroponosis, Reverse zoonosis, Flying Foxes, Zoonosis, Wildlife

1. Introduction

Zoonotic pathogens account for approximately 75% of emerging infectious diseases (EID) in humans, with spillover from wildlife to humans having been identified as one of the primary threats to global health (Taylor et al., 2001, Jones et al., 2008, Epstein and Price, 2009). As a result, emerging zoonoses are increasingly being recognised as playing a fundamental role in determining community health (John, 2013). The intricate balance between human, animal and ecosystem health has been investigated and a more holistic systems approach has subsequently been adopted under the banner of the 'One Health' initiative (John, 2013).

One still largely unexplored aspect of global heath is the potential transmission of human-borne pathogens to wildlife and domestic animals (zooanthroponosis) (Epstein and Price, 2009). Infections with zoonotic parasites, including *Cryptosporidium*, *Giardia* and *Toxoplasma* are known to be responsible for high levels of disease and morbidity in humans, but 26% of these zoonotic pathogens also infect wild and domestic animals (Current and Garcia, 1991, Taylor et al., 2001, Dubey, 2009, Feng and Xiao, 2011). As a result spillover events of these pathogens from humans into wildlife and domestic animals have the potential to cause significant and widespread disease burden, which may subsequently impact global economies and ecosystems (Coklin et al., 2007, Epstein and Price, 2009).

Cryptosporidium is recognised as one of the primary causes of diarrhoea worldwide (Fayer et al., 1997) and was identified as a 'neglected pathogen' by the World Health Organisation (WHO) in 2004. *Cryptosporidium* is primarily transmitted via contaminated food and water sources, as well as direct contact with infected humans or animals (Current and Garcia, 1991, Chalmers and Davies, 2010, Ryan et al., 2014). The two major *Cryptosporidium* spp. infecting humans are *C. hominis* and *C. parvum*, with *C. hominis* being almost entirely host specific (Morgan-Ryan et al., 2002). In contrast, *C. parvum* has been identified across a wide range of vertebrate hosts, making it an ideal candidate for an investigation into zooanthroponotic pathogen transmission (Morgan et al., 1997b, Xiao et al., 2004, Fayer, 2010)

Within Australia, habitat loss has resulted in increasing numbers of flying foxes seeking shelter in highly populated regional and urban centres (Epstein and Price, 2009). These shifts into urbanised

environments are placing mounting pressures on flying fox populations, including Grey-headed flying foxes (*Pteropus poliocephalus*) which are currently classified as threatened under the IUCN guidelines (Markus and Hall, 2004, McDonald-Madden et al., 2005, IUCN, 2014). The presence of flying foxes in urban environments further facilitates increased contact rates at the human-flying fox interface, potentially increasing the risk of pathogen spillover (Epstein and Price, 2009, Ng and Baker, 2013).

The ability of flying foxes to act as vectors for a wide variety of viral pathogens is well established, whereas their role as vectors for protozoan pathogens of human and/or veterinary importance have so far not been investigated (Epstein and Price, 2009, Ng and Baker, 2013). The presence of *Cryptosporidium* has previously been identified in a flying fox (bat genotype I) and microbat from China (bat genotype II) (Wang et al., 2013), with two additional bat genotypes having been identified in microbats from the US (bat genotype III) and from the Czech Republic (bat genotype IV) (Kvac et al., 2015). The endoparasitic fauna of Australian flying foxes has so far not been investigated.

1.1 Biology of Cryptosporidium

1.1.1 Cryptosporidium taxonomy and species identification

Cryptosporidium is a protozoan parasite that belongs to the family Cryptosporidiidae within the phylum Apicomplexa (Clark, 1999). Traditionally, *Cryptosporidium* was placed within the class Coccidea within the order Eimeriida, but phylogenetic investigations indicate that it is more closely related to the gregarines (Corliss, 1994, Carreno et al., 1999). Originally considered an obligate intracellular parasite, it has now been shown that *Cryptosporidium* can in fact replicate extracellularly, further supporting its placement within the gregarines (Hijjawi et al., 2004). Historically, *Cryptosporidium* species were named after the host from which they were initially isolated, but this was shown to be problematic as a number of species appear to have broad host ranges (Xiao et al., 2004). Variations in vertebrate host classes, oocyst morphology and infection sites amongst *Cryptosporidium* species have also been observed (Xiao et al., 2004). The wide-ranging genetic diversity of this parasite indicates that host-parasite co-evolution and host adaptation are major drivers in the heterogeneic nature of *Cryptosporidium* (Xiao et al., 2002).

Cryptosporidium oocysts lack distinguishing morphological characteristics, making species identification via microscopy unreliable (Fall et al., 2003). Molecular techniques however have proven to be a vital tool for the detection and differentiation of *Cryptosporidium* at the species

and genotype level (Xiao and Ryan, 2004, Fayer, 2010). Within this framework nested-PCRs are commonly employed for amplification of target DNA, followed by Sanger sequencing (Mayer and Palmer, 1996). Characterisation of *Cryptosporidium* can be achieved via sequence analysis across a variety of loci, including 18s rRNA, actin, heat shock protein 70 (HSP70) and glycoprotein 60 (*gp60*) (Morgan et al., 1997a, Sulaiman et al., 1999, Xiao et al., 1999b, Sulaiman et al., 2002, Xiao, 2010). The multi-copy 18srRNA locus is frequently targeted as it contains both semi-conserved and hyper-variable regions, enabling the design of genus-specific primers (Xiao, 2010). A ~298 bp region of the 18s rRNA locus has been successfully targeted for *Cryptosporidium* detection, followed by a PCR-RFLP tool targeting a ~830 bp region of the same gene for genotyping purposes, followed by subtyping at other loci (Xiao et al., 1999b, Xiao et al., 2001, Xiao, 2010).

1.1.2. Cryptosporidium: host specificity and cryptosporidiosis

Despite *Cryptosporidium* being first described in 1907, it was not until 1976 that the first case of human cryptosporidiosis was reported (Tyzzer, 1907, Meisel et al., 1976). *Cryptosporidium* has since been identified in over 150 mammalian species, as well as in birds, fish, reptiles and amphibians (Fayer, 2008). There are currently 27 recognised species of *Cryptosporidium*, seventeen of which have been reported in humans (Ryan et al., 2014) (Table 1.). The seventeen species reported in humans so far are all of homeothermic origin, only six of which are considered to be of human health importance, with *C. hominis* and *C. parvum* being the primary cause of infection (Chalmers and Katzer, 2013, Ryan et al., 2015).

The majority of *Cryptosporidium* spp. appear to be highly host adapted (Thompson et al., 2005). While some *Cryptosporidium* species are restricted to only one host type, e.g. *C. baileyi* in chicken, other species may have a slightly broader host range, e.g. *C. galli* in birds (Current et al., 1986, Pavlasek, 1999, Thompson et al., 2005) (Table 1.). In addition numerous species have been shown to infect a wide variety of vertebrate hosts (Feng, 2010, Xiao, 2010, Waldron et al., 2011b, Ryan and Power, 2012).

Globally, *C. hominis* and *C. parvum* have been identified as the primary cause of cryptosporidiosis in humans, but infections with *C. meleagridis*, *C. felis*, *C. canis*, *C. ubiquitum* and *C. cuniculus* have also been reported (Pieniazek et al., 1999, McLauchlin et al., 2000, Chalmers et al., 2009, Fayer et al., 2010). Within Australia, six *Cryptosporidium* spp. have so far been identified in humans (*C. hominis*, *C. parvum*, C*. meleagridis*, *C. andersoni*, *C. bovis* and *C. fayeri*) (Morgan-Ryan et al., 2002, Jex et al., 2008, Ng et al., 2010, Waldron et al., 2010, Waldron et al., 2011a).

1.1.3. Cryptosporidium lifecycle

The life cycle of *Cryptosporidium* is comprised of six major developmental stages (Current and Garcia, 1991) . The life cycle is completed within 3-5 days within a single vertebrate host and culminates in the shedding of infective, environmentally resistant oocysts, measuring ~ 5 µm in diameter, in the faeces of the host (Current and Garcia, 1991, Meinhardt et al., 1996, Clark, 1999) (Figure 1).

The ingestion of an infective oocyst by a new host initiates the life cycle of *Cryptosporidium*. The first stage is excystation followed by the release of 4 motile sporozoites (Current and Garcia, 1991, Meinhardt et al., 1996). The sporozoites subsequently invade the host's epithelial cells, initiating an asexual cycle, including differentiation into trophozoites and two stages of merogony (Meinhardt et al., 1996, Fayer, 2004). This is followed by the sexual cycle which initiates the formation of macro- and microgamonts, resulting in fertilisation which results in zygote formation (Meinhardt et al., 1996, Hijjawi et al., 2004).

Zygotes develop into two types of oocysts, with thin-walled oocyst recirculating within the intestinal tract of the host resulting in autoinfection, while the thick-walled oocysts are shed in the host's faeces (Current and Navin, 1986, Meinhardt et al., 1996).

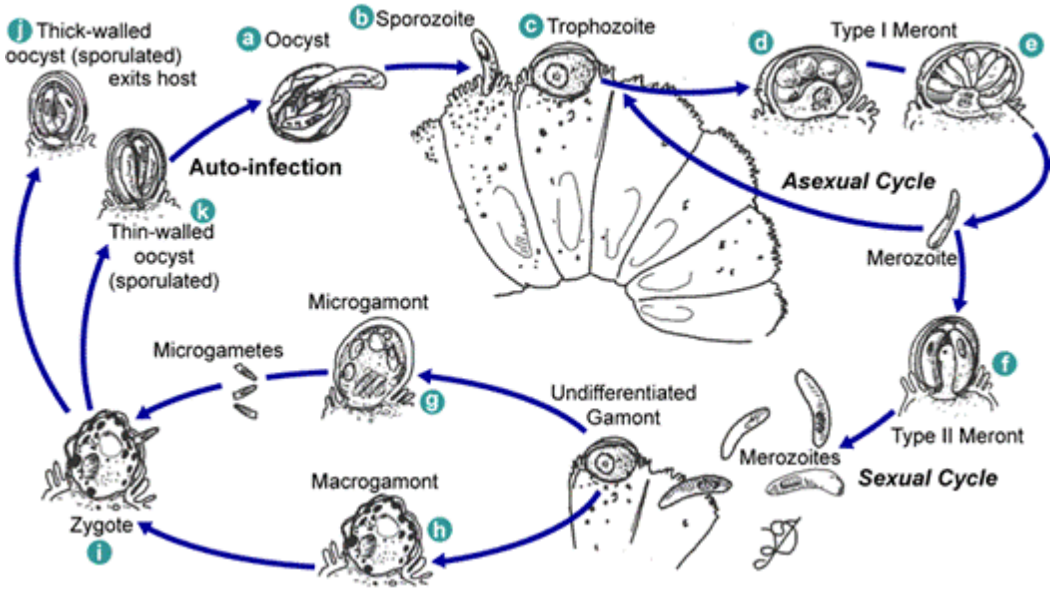

Figure 1. *Cryptosporidium* lifecycle (http://www.cdc.gov/parasites/crypto/biology.html)

1 Table 1. *Cryptosporidium* sp. identified in animals and humans

Species name	First Identified	Type host	Primary host	Reported in humans
C. muris	Tyzzer, 1907	Mouse (*Mus musculus*)	Rodents	Yes
C. parvum	Tyzzer, 1912	Mouse (*Mus musculus*)	Ruminants	Yes
C. meleagridis	Slavin, 1955	Turkey (*Meleagris gallopavo*)	Birds and humans	Yes
C. wrairi	Vetterling et al., 1971	Guinea pig (*Cavia porcellus*)	Guinea pig	No
C. cuniculus	Inman and Takeuchi, 1979	Rabbit (*Oryctolagus cuniculus*)	Rabbits	Yes
C. felis	Iseki, 1979	Cat (*Felis catis*)	Cats	Yes
C. serpentis	Levine, 1980	Snakes (*Elaphe guttata*, *E. subocularis, Sanzinia madagascarensus*)	Lizards and snakes	No
C. baileyi	Current et al., 1986	Chicken (*Gallus gallus*)	Chicken	No
C. varanii	Pavlasek et al., 1995	Emerald monitor (*Varanus prasimus*)	Lizards	No
C. galli	Pavlasek, 1999	Birds (*Gallus gallus*, *Tetrao urogallus*, *Pinicola enucleator,* Spermestidae, Frangillidae)	Birds	No
C. andersoni	Lindsay et al., 2000	Bovine (*Bos taurus*)	Cattle	Yes
C. canis	Fayer et al., 2001	Dog (*Canis familiaris*)	Dogs	Yes
C. molnari	Alvarez-Pellitero and Sitjà-Bobadilla, 2002	Fish (*Sparus aurata*, *Dicentrarchus labrax*)	Fish	No
C. hominis	Morgan-Ryan et al., 2002	Human (*Homo sapiens*)	Humans	Yes
C. suis	Ryan et al., 2004	Bovine (*Sus scofa*)	Pigs	Yes

C. bovis	Fayer et al., 2005	Bovine (*Bos taurus*)	Cattle	Yes
C. fayeri	Ryan et al., 2008	Kangaroo (*Macropus rufus*)	Marsupials	Yes
C. ubiquitum	Fayer et al., 2010	Bovine (*Bos taurus*)	Rodents, ruminants and primates	Yes
C. fragile	Jirků et al., 2008	Toad (*Duttaphrynus melanostictus*)	Toads	No
C. macropodum	Power and Ryan, 2008	Kangaroo (*Macropus giganteus*)	Marsupials	No
C. ryanae	Fayer et al., 2008	Cattle (*Bos taurus*)	Rodents, ruminants, primates	No
C. xiaoi	Fayer et al., 2010	Sheep (*Ovis aries*)	Goats and sheep	Yes
C. tyzzeri	Ren et al., 2012	Mouse (*Mus musculus*)	Rodents	Yes
C. viatorum	Elwin et al., 2012	Human (*Homo sapiens*)	Humans	Yes
C. scrofarum	Kváč et al., 2013	Bovine (*Sus scofa*)	Pigs	Yes
C. erinacei	Kváč et al., 2014	Hedgehog (*Erinaceus europaeus*)	Hedgehogs and horses	Yes
C. huwi	Ryan et al., 2015	Guppy (*Poecilia reticulata*)	Ornamental fish	No

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1.1.4. Sources and transmission pathways

Cryptosporidium is primarily transmitted through the faecal-oral route, with infected individuals shedding oocysts for a period of up to five weeks (Diers and McCallister, 1989, Fayer et al., 2000). The oocysts are environmentally resistant and can remain infectious for a number of months, depending on the environmental conditions (Anderson, 1985, Fayer et al., 1998). Transmission can occur either directly via human to human, animal to human or animal to animal contact, or indirectly via ingestion of infective oocysts in contaminated food or water sources as well as through inhalation (Ungar, 1990, Levine et al., 1991, DuPont et al., 1995, Okhuysen et al., 1999). The anthroponotic *C. hominis* has primarily been implicated in human to human infections, with *C. parvum* being responsible for zoonotic and/or anthroponotic transmissions (Tzipori, 1983, Morgan-Ryan et al., 2002).

Contamination of recreational and/or drinking water with *Cryptosporidium* oocysts has been identified as a major source of cryptosporidiosis in humans (Rose et al., 1991, DuPont et al., 1995). Oocysts are highly resistant to chemical disinfectants, including chlorine, and standard water treatment regimens are therefore ineffective at controlling waterborne outbreaks (Isaac-Renton et al., 1987, Carpenter et al., 1999). As a result cryptosporidiosis has been identified as a leading cause of diarrhoea in 1-3 % of cases in the developed world and in approximately 10% of cases in the developing world (Guerrant, 1997).

1.*2 Zooanthroponotic pathogen transmission in wildlife and livestock*

The movement of pathogens from humans into wildlife populations has wide ranging implications from a conservation and biodiversity standpoint, with a number of endangered species having been affected (Graczyk et al., 2001, Graczyk et al., 2002a, Ash et al., 2010, Hussain et al., 2013) (Table 2.). The risk of zooanthroponosis appears to be particularly high in areas affected by urbanisation, logging and hunting, consequently facilitating increased contact rates at the human/wildlife interface (Chapman, 2005).

The risk of disease spillover from humans into wildlife affects a large number of animal with waterborne enteric pathogens of potentially human origin, specifically *Cryptosporidium* and *Giardia*, having been identified in canids, pinnipeds and Australian marsupials (Ash et al., 2010, Ryan and Power, 2012, Delport et al., 2014). Incidences of infection with *Cryptosporidium* have equally been reported in 16 Australian marsupial species reviewed in Power, 2012. The two *Cryptosporidium* species known to infect humans, *C. parvum* and/or *C. hominis,* have been

reported in kangaroos, wallabies and possums (Hill et al., 2008), as well as *C. hominis/parvum* like infections in bandicoots (Dowle, 2012). It remains unclear whether these findings indicate the potential for zooanthroponotic infection in these animals, as these results are solely based on amplification at the 18s rRNA locus, with other loci not being amplifiable (Ryan and Power, 2012). Zoo environments have also been implicated in zooanthroponotic pathogen transmission, with the presence of *Trichuris trichuria* and *Ascaris lumbricoides* ova in non-human primates indicating the potential for cross-species transmission (Adejinmi and Ayinmode, 2008).

Cryptosporidium and *Giardia* have been identified as the most prevalent enteric pathogens in domestic animals, with dairy and beef cattle, especially young calves, harbouring particularly high infection rates (Thompson et al., 2008, Dixon et al., 2011). High animal densities in concentrated animal feeding operations facilitate the rapid spread of pathogens through affected populations, with close and frequent contact between livestock and humans also raising the potential for zoonotic spillover (Graham et al., 2008). One investigation into pathogens of livestock found evidence that 243 (39.4%) out of 616 were capable of infecting humans, while 335 (54.4%) were capable of infecting wildlife and 174 (28.2%) were able to infect all three (Cleaveland et al., 2001) (Table 2.). Notably, 553 (39.1%) of 1415 pathogens of human origin, were shown to infect domestic animals (including livestock), with 373 (26.4%) also infectious to wildlife and 620 (43.8%) were again capable of infecting all three categories (Cleaveland et al., 2001).

1.3 Bats as reservoirs for zoonotic pathogens

Bats are the second most abundant, species rich and geographically widespread mammal group on earth (Ng and Baker, 2013). Bats represent approximately 25% of all living mammals, with over 1232 extant species spread across every continent, excluding the polar regions (Kasso and Balakrishnan, 2013, Ng and Baker, 2013). Globally, bats are of high economic value due to their role in ecosystem services such as pollinators, seed dispersers, insect predators and bioindicators (Fujita and Tuttle, 1991, Jones et al., 2009). Despite their ubiquitous nature and wide geographic distribution bats are still considered amongst the least studied mammalian groups (Ng and Baker, 2013). Since their role as vectors for zoonotic pathogens has been identified investigations into their ecology and immune function have consequently been expanded (Kasso and Balakrishnan, 2013, Ng and Baker, 2013).

Table 2. Reports of potential anthropogenic parasite transmission in wildlife and livestock

Parasite Phylum	Pathogen species	Infected animal(s) type/location	Type Location/ Country	Reference
Nematoda	*Chilomastix mesnili, Endolimax nana, Stronglyoides fuelleborni, Trichuris trichiura*	Mountain gorillas (*Gorilla gorilla beringei*)	National Park Rwanda, Africa	Sleeman et al., 2000
Apicomplexa	*C. parvum*	Mountain gorillas (*Gorilla gorilla beringei*)	National Park Uganda, Africa	Graczyk et al., 2001
Microspora	*Encephalitozoon intestinalis*	Mountain gorillas (*Gorilla gorilla beringei*)	National Park Uganda, Africa	Graczyk et al., 2002a
Apicomplexa	*G. duodenalis*	Mountain gorillas (*Gorilla gorilla beringei*)	National Park Uganda, Africa	Graczyk et al., 2002b
Apicomplexa	*C. parvum*	Calf & mouse^{+}	Laboratory study Korea	Guk et al., 2004
Heterokontophyta	*Blastocystis* sp.	Various animal hosts	Laboratory study	Noël et al., 2005
Apicomplexa	*G. duodenalis, C. parvum*	Dairy Cattle	Farm Ontario, Canada	Coklin et al., 2007
Nematoda	*Ascaris lumbricoides, T. trichiura*	20 animal sp.	Zoo Nigeria, Africa	Adejinmi and Ayinmode, 2008
Apicomplexa	*C. parvum*, *C. hominis* like	Common brushtail possum (*Trichosurus vulpecula*)*	Free-ranging in Zoo/ free-ranging in non-urban reserve Sydney, Australia	Hill et al., 2008
Apicomplexa	*Isospora spp., Giardia duodenalis*	Colobus monkey (*Colobus vellerosus*)	Boabeng-Fiema Monkey Sanctuary Ghana, Africa	Teichroeb et al., 2009

Apicomplexa	*G. duodenalis*	African painted dog (*Lycaon pictus*) (Wildlife; Zoo)	Wild populations Zambia & Namibia, Africa; Zoo Australia	Ash et al., 2010
Apicomplexa	*G. duodenalis*	Red colobus monkeys & Livestock National park	National Park Uganda, Africa	Johnston et al., 2010
Apicomplexa	*C. parvum* *C. parvum, C. hominis*	Wallaby (sp. not identified) Eastern grey kangaroos (*Macropus giganteus*)	Sydney, Australia	Ng et al., 2011
Apicomplexa	*G. duodenalis, C. parvum*	Beef & Dairy cattle	Farm Ontario, Canada	Dixon et al., 2011
Apicomplexa	*C. parvum/hominis* like	Long-nosed bandicoot (*Perameles nasuta*) Southern Brown Bandicoot (*Isoodon obesulus*)	Australia	Dowle, 2012
Apicomplexa	*G. duodenalis*	Australian sea lions (*Neophoca cinerea*)	Australia	Delport et al., 2014
Apicomplexa	*G. duodenalis*	Brush-tailed rock-wallaby (*Petrogale penicillata*)	Wild & captive populations NSW, Australia	Vermeulen et al., 2015b

*indicating the potential for zooanthroponotic infection only, require further investigation at additional loci

[+]indicating experimental infection

A vital factor underpinning the role of bats as reservoir hosts for zoonotic pathogens lies in the relatively early evolution of the order Chiroptera, and their divergence into the Mega- and Microchiroptera approximately 52 million years ago (Teeling et al., 2005). The two lineages have remained relatively unchanged since their first appearance, indicating a longstanding co-evolutionary history with a number of ancient pathogens (Hill, 1984, Calisher et al., 2006).

It has now been recognised that ecological changes resulting from habitat alterations drive the emergence of novel infectious diseases, as a result of altered host-parasite interactions, consequently threatening public health, as well as global biodiversity (Kasso and Balakrishnan, 2013, Luis et al., 2013). Habitat loss, encroachment and hunting facilitate increased contact between wildlife, humans and livestock, further promoting the spread of these pathogens (Ng and Baker, 2013).

Research efforts have been highly focused on pathogens known to directly impact human health, including *Trypanosoma* and *Plasmodium* (Woo and Hawkins, 1975, Hamilton et al., 2012, Lima et al., 2012, Schaer, 2013), with a smaller number of studies investigating the intestinal helminths of bats (Wolfgang, 1954, Jameson, 1959, Cain, 1966, Ubelaker, 1966, Bundy and Bundy, 1988, Prociv, 1989, Prociv, 1990). Considering the role of bats as disease vectors for pathogenic viruses, other endoparasitic infections harboured by bats may also be capable of crossing the species barrier, resulting in spillover events that could threaten both livestock and human health (Kuzmin et al., 2011).

The ability of bats to harbour a wide range of zoonotic pathogens also appears to be closely linked to their life history and ecology (Luis et al., 2013). Firstly, their highly gregarious nature facilitates increasing levels of inter- and intraspecific contact between individuals, with roosts often housing diverse species assemblages, some of which contain well over a million individuals (Luis et al., 2013). The highly varied population structure observed within the Chiroptera also allows for elevated inter- and intraspecific contact rates which promote accelerated transmission of pathogens amongst individuals. The roost ecology of bats, coupled with their ability to disperse over large areas therefore place bats in an ideal position as vectors for both new and re-emerging infectious diseases (Luis et al., 2013).

1.4 Australian flying foxes

Australia hosts a total of 77 bat species, including eight species of pteropodid bats, commonly known as flying foxes or fruit bats (McKay et al., 1989). Australian flying foxes inhabit tropical and

subtropical zones and are distributed predominantly along the coastal regions of Victoria, NSW, QLD, the Northern Territory and Western Australia (Markus and Hall, 2004, McDonald-Madden et al., 2005, Van Der Ree et al., 2006) (Figure 2.).

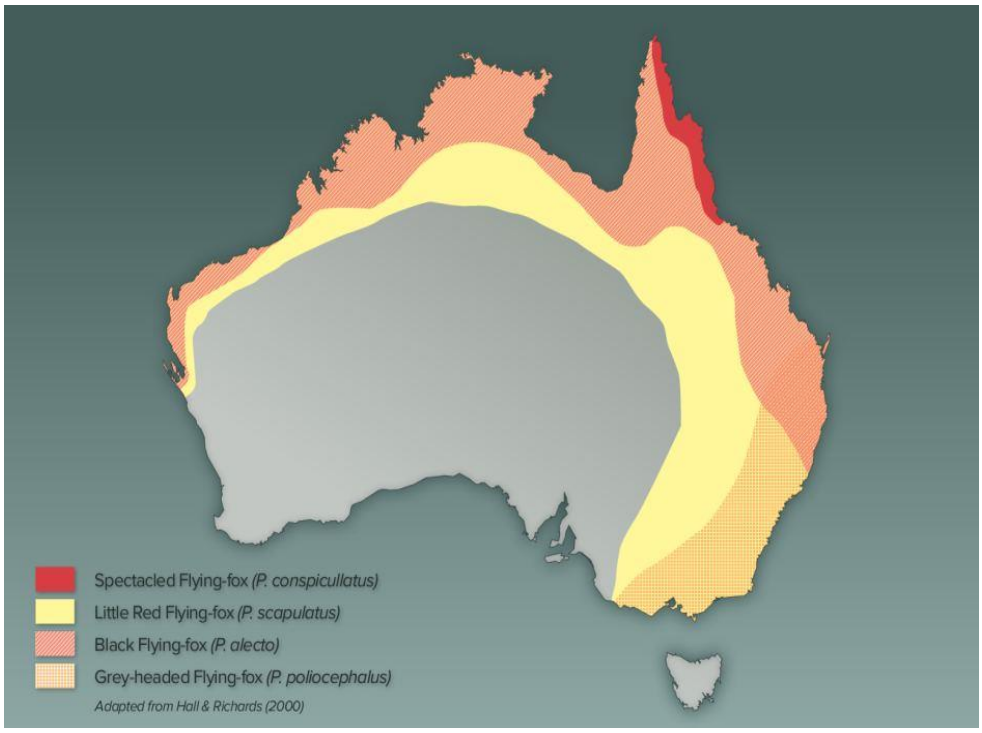

Figure 2. Australian flying fox distribution (www.abc.net.au)

Flying foxes rely heavily on seasonal fruit and nectar food sources which are widely dispersed in native forests (Hall and Richards, 2000). The decline of these natural food sources as a consequence of anthropogenic impacts has thus resulted in the movement of these animals into more highly populated areas where native gardens and parks provide reliable alternative food sources (Parry-Jones and Augee, 2001, Williams et al., 2006). This has led to an increase in urban populations, with most major cities along Australia's east coast now hosting continuously occupied flying fox roosts (Markus and Hall, 2004, McDonald-Madden et al., 2005, Van Der Ree et al., 2006).

With a number of Australia flying fox species listed as vulnerable or endangered under the IUCN guidelines, the majority of scientific studies have focused on their ecology, distribution and dispersal capacity in order to develop adequate management strategies (Fujita and Tuttle, 1991, Hall and Richards, 2000, Snoyman et al., 2012, Ng and Baker, 2013, IUCN, 2014).

Despite the fact that only a limited number of potentially infectious parasite genera have been identified in flying fox populations so far, this number is bound to increase as human-bat interactions increase and investigative efforts are expanded (Table 3.). Captive Grey-headed flying

foxes (*P. poliocephalus*) in Sydney for example, were found to harbour the parasitic nematode *Angiostrongylus cantonensis* (Reddacliff et al., 1999). This nematode causes neurological disease in a number of animals, including humans and was also responsible for disease outbreak in dogs within the Sydney city region (Reddacliff et al., 1999). Australian flying foxes have also been shown to harbour protozoan parasites including species of *Trypanosoma*, *Toxoplasma* and *Hepatocystis* (Mackerras, 1958, Mackerras, 1959, Sangster et al., 2012). The presence of these protozoan parasites has important implications for Australia's endemic fauna, livestock and human health, as host switching or spillover events could potentially lead to infections and ultimately disease outbreaks (Mackerras, 1958, Prociv, 1983, Reddacliff et al., 1999, Barrett, 2002, Schaer, 2013) .

The first case of *Cryptosporidium* in a bat was identified in a tissue sample of a microbat (*Eptesicus fuscus*) in 1998 (Dubey et al., 1998), followed by the identification of a *Cryptosporidium* "mouse" genotype in faecal samples of another microbat species (*Myotis adversus*) one year later (Morgan et al., 1998, Morgan et al., 1999a). The presence of *Cryptosporidium* in a megabat (*Rousettus leschenaultia*) from China was recently confirmed and represents the only report of *Cryptosporidium* in megabats to date (Wang et al., 2013). With at least eight species of *Cryptosporidium* known to cause disease in humans, the potential presence of this pathogen in Australian flying fox populations should be investigated (Ryan et al., 2014).

1.5 Impacts of ecological alterations on pathogen transmission

Habitat fragmentation and degradation, as a consequence of anthropogenic environmental changes, are having wide ranging impacts on global biodiversity and ecosystem health (Cottontail et al., 2009). One such impact is the alteration of the gastrointestinal parasite profile of animals, as a consequence of altered host-parasite interactions (Hussain et al., 2013). The resulting alterations in parasite profiles are primarily driven by changes in host density which consequently influence parasite community structure and prevalence within hosts.

Host density is known to influence species richness and prevalence of directly transmitted parasites, with increases in host density resulting in increased transmission rates of parasites and multi-host parasitism due to increased contact rates (Cottontail et al., 2009, Hussain et al., 2013). Increases in contact rates are particularly relevant in group living species, as group size corresponds to host density (Hussain et al., 2013, Luis et al., 2013). Habitat fragmentation and degradation have been identified as one of the primary drivers of biodiversity loss (Sih et al., 2000). Fragmentation significantly reduces species ranges, while also increasing contact rates

between populations, resulting in 'edge effects' (Ash et al., 2010). Edge effects alter the incidence and effect of pathogens on these populations in a number of ways, depending on the underlying host-pathogen interaction (McCallum and Dobson, 2002). First, transmission rates of host-specific pathogens may decline as a result of edge effects, as the pathogen becomes effectively 'quarantined' within the infected patch as dispersal rates of individuals between groups are reduced. This applies particularly in single-pathogen-single-host systems, which are highly density dependant (McCallum and Dobson, 2002). Alternatively, a pathogen may persist in a reservoir host or host complex. The pathogen may therefore occupy the patch itself or, as is often the case with livestock, the matrix around the patch or both. The presence of reservoir hosts consequently results in more complex disease dynamics which are highly dependent on the balance between both patch colonisation and extinction (McCallum and Dobson, 2002).

1.6 Study aims

The aim of this study was to investigate the potential for anthropozoonotic pathogen transfer of human-borne *Cryptosporidium* spp. into Australian flying fox populations. Samples from wild and captive flying fox populations in New South Wales (NSW) and Queensland (QLD) were collected and molecular methods were employed in order to explore *Cryptosporidium* prevalence and diversity.

I hypothesise that captive flying fox populations will have a higher prevalence of infection with human-borne *Cryptosporidium* spp. when compared to wild populations as a consequence of increased contact rates due to handling and feeding.

Table 3. Protozoan parasites identified in megabats

Table showing parasite phylum and species identified in megabats by geographic region

Phylum	Species	Megabat species	Location	Reference
Apicomplexa	*Hepatocystis pteropi*	*Pteropus conspicullatus* *Pteropus colinus* *Pteropus geddiei* *Pteropus gouldii* *Pteropus poliocephalus* *Pteropus scapulatus*	Australia New Guinea	Mackerras, 1958
Apicomplexa	*Toxoplasma gondii*	*Pteropus conspicillatus* *Pteropus scapulatus*	Australia	Sangster et al., 2012
Apicomplexa	*Plasmodium* sp. *Hepatocystis* sp.	*Myonycteris angolensis* *Epomophorus gambianus* *Epomops buettikoferi* *Hypsignathus monstrosus* *Micropteropus pusillus* *Myonycteris leptodon* *Nanonycteris veldkampii* *Eidolon helvum* *Megaloglossus azagnyi* *Rousettus aegyptiacus* *Scotonycteris ophiodon* *Scotonycteris zenkeri*	West Africa	Schaer, 2013
Apicomplexa	*Cryptosporidium* bat genotype 1 & 2	*Rousettus leschenaultia*	China	Wang et al., 2013

2 Materials and methods

2.1 Sites and sample collection

Faecal samples (*n* = 281) from nine FF populations inhabiting the east coast of Australia were collected for parasite analysis. The sample populations consisted of urban and rural sites in NSW (Centennial Park, North Avoca, Singleton, Tocal, Port Macquarie, Byron Bay, Gordon) and two captive populations, one in NSW (Matcham) and one in QLD (Tolga) (Figure 3. & Table 4.). All wild population samples (*n* = 179) were opportunistically collected from Grey-headed flying fox (*P. poliocephalus*) camps in 2012 and 2013. Captive NSW samples (*n* = 78) were collected from *P. poliocephalus* held at the Wambina Flying Fox Sanctuary (a re-release facility operated by the Wildlife Animal Rescue and Care Society Inc.), located 55 kilometres north of Sydney in February and March 2015. Plastic sheets were placed below the roosting areas prior to feeding, followed by sample collection. Captive QLD samples (*n* = 24) were collected in April 2015 at the Tolga Bat Hospital located in the Atherton Table Lands near Cairns. The Tolga population consisted primarily of Spectacled and Little Red flying foxes (*P. conspicullatus* and *P. scapulatus* respectively), with only one individual each of Black- (*P. alecto*) and Grey-headed flying foxes (*P. poliocephalus*). The animals were found to aggregate in small single species groups allowing samples to be collected from their respective feeding areas. In order to minimise the risk of pseudoreplication within the captive populations only non-neighbouring samples (distances > 15cm) were collected.

Faecal samples were stored for further processing at 4˚C. The samples were irradiated by exposure to a ^{60}Cobalt source for 35 mins prior to DNA extraction in order to reduce their pathogenic load.

Figure 3. Sampling locations for wild and captive flying fox populations in NSW and QLD Map showing locations of wild sample populations in NSW which included Sydney, North Avoca, Tocal, Singleton, Port Macquarie and Byron Bay. Captive NSW samples were collected in Matcham and Tolga, QLD.

2.2 DNA extraction and PCR screening for Cryptosporidium characterisation

DNA was extracted from faecal material (~ 150 mg) using the Isolate Fecal DNA kit (Bioline, London, UK). Faecal material was weighed into the lysis bead tubes and DNA was extracted following the manufacturer's instructions. Extracted DNA was stored at – 20˚C until further processing.

Prior to PCRs DNA samples (*n* = 281) were combined with Gene Releaser (5 µl) (BioVentures, Inc., TN, USA) and overlaid with paraffin oil (Biotech Pharmaceuticals PTY LTD, Australia) before microwaving on high (7 min) in a 500 W microwave. Samples were screened for *Cryptosporidium* using a nested PCR protocol targeting a small fragment (~ 298 bp) of the 18sRNA gene. DNA amplification was performed using the forward and reverse primers LXF1/LXR1 (Supplementary 1.) following the methodology of Xiao et al. (1999), but the concentration of $MgCl_2$ was lowered to 2 mM. The secondary amplification used the primers 18S IF/18S IR and followed the methodology of Morgan et al. (1997). Primary PCR reactions were performed in 50 µl and secondary reactions in 25 µl.

In order to further increase specificity for *Cryptosporidium* both reactions were modified by lowering dNTP concentrations to 50 µM (Vermeulen et al., 2015a). All PCR amplifications were performed using Red Hot Taq DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA) (Hill et al., 2008) and were carried out in an Eppendorf Mastercycler (Eppendorf, North Ryde, Australia), following the methodology of Vermeulen et al. (2015).

All secondary PCR products produced during this study were visualised by agarose gel electrophoresis (2%) using TBE and SYBR Safe staining (2 µl) (Promega, Australia). Bands were compared to a Hyperladder II DNA marker to identify product size (Bioline, London, UK). Amplicons of correct size were considered to be positive. List of primers and primer sequences used during PCR are provided (Suppl. 1.).

Putative *Cryptosporidium* positive samples were used to generate longer 18s rRNA fragments (~ 825 bp) using primary product (2 µl) from the original reaction (described above). Amplification of longer fragments were performed in 50 µl volumes using the primers LXF2/LXR2 developed by Xiao et al. (1999) and following the methodology of Waldron et al. (2011).

2.3 PCR screening at the actin locus

Isolates positive at the longer 18s rRNA fragment were screened at a second locus, targeting actin to confirm *Cryptosporidium* species identity. Amplification of the actin locus (~ 800 bp) was performed using a nested PCR (to be referred to as actin 1) following the protocol of Suleiman et al (2002). Minor modifications to this protocol were applied in order to increase specificity for *Cryptosporidium*. The concentrations of $MgCl_2$ and dNTP were lowered to 2 mM and 50 µM respectively and the secondary PCR annealing temperature was raised to 54˚C. Primary PCRs were performed in 25 µl, followed by secondary reactions in 50 µl.

Prior to the actin 1 PCR, DNA samples were combined with Gene Releaser (5 µl) (BioVentures, Inc., TN, USA) and overlaid with paraffin oil (Biotech Pharmaceuticals PTY LTD, Australia) as described above. PCRs were performed in 50 µl using Red Hot Taq DNA polymerase. Primary reaction primers were Act F1/Act R1, with secondary primers consisting of Act F2/Act R2 (Sulaiman et al., 2002).

2.4 Additional screening at the actin locus

An additional protocol targeting the actin locus was applied (Ng et al., 2006) to all *Cryptosporidium* positive 18s rRNA isolates in order to resolve inconsistent amplification with the above protocol

(to be referred to as actin 2). A nested PCR was performed with minor modifications. Primary PCR was performed using the forward and reverse primers AII F1/Act6R targeting a ~830 bp product of the actin locus. The dNTP concentrations were lowered to 50 µM and PCR cycles were reduced from 50 to 40 cycles in order to increase specificity for *Cryptosporidium*. The secondary PCR consisted of the forward and reverse primers AII F2/AII R1 targeting a ~818 bp fragment of the actin locus. Concentrations and PCR conditions were identical to the primary protocol.

2.5 PCR screening at the glycoprotein 60 (gp60) locus

The *gp60* locus was amplified using a nested PCR protocol. Primary reaction primers were outF/outR targeting a 1442bp fragment, with secondary primers consisting of S60.ATGF/S60.StopR targeting a smaller ~1000bp fragment. Reactions were performed in 50 µl following the methodology of Waldron et al. (2009).

2.6 PCR purification protocols

All positive PCR amplicons were purified using either the Qiaquick or MinElute PCR Purification Kit (Qiagen), depending on the intensity of the gel bands (produced during electrophoresis) in preparation for cloning and sequencing.

2.7 DNA sequencing and sequence analysis

Positive amplicons generated for the 18s rRNA locus (~ 825 bp), actin 1, actin 2 and *gp60* were sequenced in order to facilitate *Cryptosporidium* species identification. Sequencing was performed using the appropriate primers in the forward and reverse direction (Macrogen, Seoul, Korea). Sequences were aligned and manually checked for quality and read errors and consensus sequences were extracted using Geneious (version 8.1.3, Biomatters LTD, New Zealand).

2.8 Cryptosporidium 18s clone library

Cloning of *Cryptosporidium* 18s positive samples was performed to obtain individual sequences from mixed PCR product, as indicated by sequencing of PCR amplicons at the 18s rRNA locus. Alignment of forward sequences from captive Matcham samples indicated a 100% sequence match between FF467 and FF469 and between FF471 and FF475, therefore two representative samples (FF467 & FF471) were chosen for cloning. Ligation of amplicons into pGEM T-Easy plasmid vectors (Promega, Madison, USA) was performed and subsequently transformed into competent One Shot Top 10 *Escherichia* coli cells (Invitrogen, Carlsbad, California). Cells were cultured on LB agar plates (Oxoid, Adelaide, Australia) containing 100 mg ampicillin/ml (Sigma-Aldrich, St Louis,

USA) and treated with 40 mg X-Gal/ml (Bioline, London, UK) and 40 µl IPTG (100 mM stock) (Bioline, London, UK). Identification of recombinant plasmids was performed via PCR screening and agarose gel electrophoresis. *Cryptosporidium* positive plasmids were purified in preparation for sequencing using the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

2.9 Statistical analysis

Chi-square test was used to determine if there was a significant difference in *Cryptosporidium* prevalence between wild and captive flying fox populations. Minitab (version 17.2.1.0, Minitab Inc.) was used for statistical analysis.

3 Results

3.1 Cryptosporidium detection

DNA was extracted from 281 faecal samples and screened for *Cryptosporidium* using 18s rRNA (~298 bp) PCR. Of the screened samples, 30 produced amplicons of the expected size and were deemed positive for *Cryptosporidium* by gel electrophoresis. Of the 30 positive amplicons nine were subsequently confirmed as *Cryptosporidium* spp. by amplification of the longer 18s rRNA fragments (~ 825 bp) and DNA sequence analysis. *Cryptosporidium* positive samples were identified in samples from one wild and two captive flying fox sites. Overall prevalence of *Cryptosporidium* was 3.2% (9/281), with a prevalence of 1.7% (3/179) in wild versus 6.3% (6/102) in captive individuals. The Tocal population (wild) had the highest prevalence of *Cryptosporidium* (20%), but the number of samples tested was the lowest (*n* = 15) (Table 4.). *Cryptosporidium* prevalence in captive individuals at Matcham and Tolga was 5.1% (4/78) and 8% (2/24) respectively. There was no significant difference in *Cryptosporidium* prevalence between wild and captive flying foxes ($\chi^2 = 3.708$, DF = 1, $p = 0.054$).

Table 4. Positive PCR results for loci tested across sampling sites.
Number of samples per site and population type indicating *Cryptosporidium* positive loci at 18s rRNA, actin and *gp60* as indicated by sequencing.

Sample site	Population type	No. of samples	18s rRNA (~825 bp)	actin 1* (~830 bp)	actin 2* (~818 bp)	*gp60*
Centennial Park[1]	Wild	15	0	0	0	0
North Avoca[1]	Wild	15	0	0	0	0
Singleton[1]	Wild	15	0	0	0	0
Tocal[1]	Wild	15	3	1	0	0
Port Macquarie[1]	Wild	40	0	0	0	0
Byron Bay[1]	Wild	49	0	0	0	0
Gordon[1]	Wild	30	0	0	0	0
Matcham[2]	Captive	78	4	1	0	0
Tolga, QLD[2]	Captive	24	2	1	0	1

* Indicates two different actin protocols which were applied at this locus
[1] Population consisting of *P. poliocephalus*
[2] Population consisting of *P. conspicullatus, P. poliocephalus, P. scapulatus* and *P. alecto*

3.2 Cryptosporidium sp. identification

Cloning and sequencing identified the presence of four novel sequences (bat genotype V-VIII) in clones from Tocal and Matcham (Figure 4). Three novel sequences (bat genotype V-VII) were identified in clones from Tocal (FF34, FF38 and FF43), with a fourth novel sequence (bat genotype VIII) being identified in all clones from Matcham (FF467, 469, 471 and 479). Sample FF34 contained two novel sequences (bat genotype V and VII), with one of these novel sequences (bat genotype VII) also being present in sample FF43. Sample FF38 contained the third novel sequence (bat genotype VI) (Table 5). Intra clone variation was minimal, with distance matrices showing that two out of three clones from both Tocal and Matcham were 97.7-99.9% similar. BlastN searches revealed that all clones matched unknown *Cryptosporidium* spp. with nucleotide matches between 96.8% and 98% (Table 6).

Cloning of captive Tolga samples identified the presence of *C. hominis* in both samples (FF614 and FF616). Four out of five clones from FF614 produced sequences which were 99.5-100% identical to *C. hominis* (BlastN), with one clone (614-8) showing more variation and producing a 99.9% match to *C. parvum*. All sequences generated from clones for sample FF616 were > 99.5% identical as

indicated by distance matrices and produced 99.9-100% matches to *C. hominis*. Both intra- and inter- sample variation for FF614 and FF616 clones was minimal and consistent (99-99.9%).

3.3 Cryptosporidium sp. confirmation targeting actin and gp60

Confirmation screening for *Cryptosporidium* at the actin locus produced two partial forward sequences matching 90% to *Cryptosporidium* sp. (BlastN) for samples FF43 and FF471 (Table 4), with one positive actin sequence for sample FF614 producing a 100% match to *C. hominis.* Screening at the *gp60* locus resulted in one positive *gp60* sequence for sample FF614, with a 99.7% match to *C. hominis* (subtype IbA9G2).

Table 5. Differences among partical 18s rDNA regions for *Cryptosporidium* bat genotypes

Comparison of *Cryptopsoridium* bat genotypes identified in flying foxes (*Pteropus*) and other bat hosts

		260	468	657	739
C. parvum		TTAA-----ATGT-GACATATCCAT	GACTTTT---TGGT	ATATAATATTAACATAATTCATATTACTATATATTT-----TAGTAT	GCATATGCCTT
Bat genotype I	ex *Rhinolophus*	TTTT-----ATGT-GACATAT-CCA	GACTTT--AACAGT	ATATAATATTAACATAATTCATATTACTATTCCTTA-----TAGTAT	GCCAATGCCTT
Bat genotype IV	ex Pipistrellus	TTTT-----TTGT-GACATAT-CAT	GACTTT--AACAGT	ATATAATATTAACATAATTCATATTACTATTCCTTA-----TAGTAT	GCCAATGCCTT
Bat genotype I	ex Aselliscus	TTTT-----ATGTTGACATATTCCC	GACTTT--AACAGT	ATATAATATTAACATAATTCATATTACTATTTTT--------AGTAT	GCTATTGCCTT
Bat genotype VIII	ex Pteropus	TATT-----TTGT-GACATAT-CAT	GACTTAT---TGGT	ATATAATATTAACATAATTCATATTACTATAT-TGT-----TAGTAT	GCAATTGCCTT
Bat genotype VII	ex Pteropus	TTTTTT---ATGT-GACATAT-CAT	GACTTTTTAATAGT	ATATAATATTAACATAATTCATATTACTATATTTACGT--CTAGTAT	GCTTTTGCCTT
Bat genotype V	ex *Pteropus*	TTTTTTTTGATGT-GACATAT-CAT	GACTTTTTAATAGT	ATATAATATTAACATAATTCATATTACTATATTTACGTGTCTAGTAT	GCTTTTGCCTT
Bat genotype VI	ex *Pteropus*	TTTTTTTT-ATGT-GACATAT-CAT	GACTTTTTAATAGT	ATATAATATTAACATAATTCATATTACTA--TTTAC----CTAGTAT	GCTTTTGCCTT
Bat genotype III	ex *Eptesicus*	TTTTAAT--ATGT-GACATAT-CAT	GACTTT--AATAGT	ATATAATATTAACATAATTCATATTACTA--TAAATTTTTTTAGTAT	GCATATGCCTT
Bat genotype II	ex Hipposideros	ATTT-----ATGT-GACATATCCAT	GGCCTC---ACGGT	AGATAAGGTTAACATACTTCATGTGCTCGTTTACG-------GGTGT	GCAATTGCCTT

Table 6. Species identification for cloned isolates
Clone isolate identities showing % similarities to *Cryptosporidium* sp. as indicated by BlastN searches.

Site	FF species	Sample ID	GenBank hit accession no.	% similarity	Type name
Tocal	*P. poliocephalus*	FF34	AF247535	96.8%	*Cryptosporidium* sp. SSU rRNA gene
			AF247535	97.3%	*Cryptosporidium* sp. SSU rRNA gene
		FF38	AF247535	97-97.4%	*Cryptosporidium* sp. SSU rRNA gene
		FF43	AF247535	97-97.4%	*Cryptosporidium* sp. SSU rRNA gene
Matcham	*P. poliocephalus*	FF467*	JQ40103	97.6-98%	*Cryptosporidium* sp. Weddell seal genotype SSU rRNA gene
		FF471*	JQ40103	97.7-98%	*Cryptosporidium* sp. Weddell seal genotype SSU rRNA gene
Tolga	*P. conspicullatus*[*2]*	FF614	KF679722	99.5-100%	*C. hominis* isolate NY-48 18S rRNA gene
			GU319779	99.5%	*C. hominis* strain gx02 18S ribosomal RNA gene
			AF112569	99.6-99.9%	*C. parvum* strain CPRM1 18S rRNA gene
	P. conspicullatus[*2]*	FF616	GU319779	99.9- 100%	*C. hominis* strain gx02 18S ribosomal RNA gene
			L16997/ DQ286403	99.9%	*C. parvum* 18s rRNA/*C. hominis* 18S ribosomal RNA gene
			AF112569	99.9%	*C. parvum* strain CPRM1 18S rRNA gene

* indicates representative samples used for cloning from identical Matcham samples (467, 469, 471, 475) as indicated by sequencing analysis

*[2] presumptive species identification based on collection data provided

4 Discussion

This study reveals the presence of *Cryptosporidium* spp. in wild and captive Australian flying foxes. Cloning and sequencing results provide evidence for zooanthroponotic pathogen transfer from humans to captive flying foxes in QLD, as well as indicating the presence of four novel genotypes across wild and captive flying foxes in NSW and QLD. *Cryptosporidium* prevalence (3.2%) is consistent with levels detected in other bat species, which range between 2.8% and 9.5% (Ziegler et al., 2007, Wang et al., 2013, Kváč et al., 2015). Prevalence of *Cryptosporidium* in bats has however been shown to vary greatly, with one US study reporting a prevalence of 57.1% in an unknown bat species (Ziegler et al., 2007). The hypothesis that captive individuals have a higher prevalence of *Cryptosporidium* than their wild counterparts was not confirmed, with no significant difference detected between these groups.

PCR targeting the short 18s rRNA fragment (~ 298 bp) is commonly applied for *Cryptosporidium* detection and species identification in faecal samples, as this methodology provides better sensitivity than microscopy (Morgan et al., 1997b, Xiao et al., 1999b, Fall et al., 2003, Ryan et al., 2008). Targeting of this fragment by did however result in a large number of false positives within this study. PCR and subsequent gel electrophoresis indicated 30 samples as being positive for *Cryptosporidium*, whereas targeting of the larger fragment (~825 bp) correctly identified all nine *Cryptosporidium* positive amplicons which were subsequently confirmed by sequencing, therefore providing a more robust method for the detection of *Cryptosporidium* in this study, as previously demonstrated by Xiao et al. (1999a).

Although the actin and *gp60* locus are widely used for detection and classification of *Cryptosporidium* species, amplification at these loci was not optimal for the majority of samples (Sulaiman et al., 2002) (Table 4.). The lack of amplification at the actin locus is likely the result of low oocyst burdens, which were reflected in low amplification signals (Hill et al., 2008). The presence of mixed species cannot be ruled out, with a number of sequences showing multiple peaks. The single copy nature of the actin (Kim et al., 1992) and *gp60* gene (Strong et al., 2000) may also have contributed to low PCR amplifications when compared to the five copy 18s rRNA gene (Le Blancq et al., 1997) within the same samples. Additionally, the lack of amplification for *gp60* in samples containing the novel genotypes is more likely due to the highly polymorphic nature of this locus (Power et al., 2009).

The finding of novel genotypes in flying foxes is not unusual, with many new *Cryptosporidium* genotypes being identified in wildlife hosts which are examined for this parasite for the first time (Ziegler et al., 2007, Ryan and Power, 2012). Three novel genotypes were identified in wild Grey-headed flying foxes population from Tocal in NSW, with the fourth new genotype being present in a captive population in Matcham, NSW.

Clones obtained from captive Tolga samples (FF614 and FF616, QLD) were identified as *C. hominis*. Differentiation of *C. hominis* and *C. parvum* at 18s rRNA can be problematic due to the high level of similarity at this locus (99.7%), but the presence of a 7-11 thymine region reliably differentiates *C. hominis* from *C. parvum* (Morgan et al., 1999, Power et al., 2011). The presence of this thymine region in both amplified sequences supports *C. hominis* as the only species within these samples. One sample (FF614) also produced a sequence with a 100% match to *C. hominis* at the actin locus. This finding was confirmed by amplification of *gp60* for this sample which produced a 99.7% match to *C. hominis*.

The identification of *C. hominis* within a hospital environment may indicate that illness and/or injury may make flying foxes particularly susceptible to secondary infections due to stress and lowered immune function, subsequently facilitating zooanthroponotic pathogen transmission (Ng and Baker, 2013). Identification of *C. hominis* in captive flying foxes highlights the fact that spillover can occur, and that these animals may consequently act as potential reservoirs and/or vectors for *C. hominis* infection. Spillover and potential spillback of this pathogen could therefore have a number of important 'One Health' implications (Kelly et al., 2009, John, 2013).

Firstly, spillover of *C. hominis* and its potential impact on flying fox health is currently unresolved and requires further investigation. Although the presence of *C. hominis* was restricted to a captive population, the fact that animals are generally released following treatment may facilitate the spread of this pathogen into wild populations. In light of the fact that flying fox populations are already under pressure from anthroponotic impacts, with Grey-headed flying foxes classed as threatened, spillover may further impact the survival of these populations (Markus and Hall, 2004, McDonald-Madden et al., 2005, IUCN, 2014).

Secondly, the risks associated with zooanthroponosis may extend beyond the potential impacts on flying fox populations (Kelly et al., 2009). Spillback, whereby the human-borne *Cryptosporidium* sp. which moved into the flying fox population is cycled back into human hosts must also be considered (Epstein and Price, 2009). The foraging ecology of flying foxes, coupled with their

increased presence in regional and urban centres, particularly near orchards and private gardens may for example facilitate contamination of fruit and vegetables with human pathogenic *Cryptosporidium* oocysts (Markus and Hall, 2004, Williams et al., 2006). Considering that *Cryptosporidium* was the causative agent in over 60% of global waterborne protozoan disease outbreaks between 2004 and 2010, spillback could have further implications for human and/or livestock health (Epstein and Price, 2009, Kelly et al., 2009, Baldursson and Karanis, 2011). With the role of wildlife as a source of water contamination with *Cryptosporidium* oocysts well established, it is clear that the presence of *C. hominis* in flying foxes may equally contribute to contamination of recreational and drinking water sources (Parry-Jones and Augee, 2001, Jiang et al., 2005, Alves et al., 2006). The fact that *Cryptosporidium* can be transmitted across a variety of host species is equally of concern from a conservation perspective, as the majority of recent disease outbreaks in endangered species were the result of multi-host pathogens (Cleaveland et al., 2001). Both anthroponotic and zoonotic pathogen spillover of *Cryptosporidium* spp. therefore has the potential to further endanger Australia's unique endemic fauna and may consequently result in further loss of global biodiversity (Epstein and Price, 2009).

Future research is required to determine whether the novel *Cryptosporidium* genotypes identified in Grey-headed flying foxes in this study are also present in other flying fox species outside of NSW. Studies should be expanded to include sampling localities across states and territories, incorporating the entire home range of endemic Australian flying foxes. Larger sample sizes would also be beneficially in providing a more accurate assessment of *Cryptosporidium* prevalence within and across flying fox species. Further investigations into the level of zooanthroponotic transmission of *Cryptosporidium* in captive environments would also be beneficial as it may provide a better understanding of the underlying pathways and help inform best practice in relation to animal handling and feeding. Since anthroponotic pathogens are most commonly reported in domestic livestock and captive animals where contact rates with humans are particularly high, it follows that increased contact at the human/wildlife boundary may equally facilitate this process (Epstein and Price, 2009). Distance between flying fox roosts and human settlements may therefore be another factor playing a role in infection of flying foxes with human-borne *Cryptosporidium* and this relationship should therefore be investigated.

The potential for zoonotic and anthroponotic pathogen transmission within an Australian context clearly requires further investigation. A large number of native species have not yet been investigated in relation to their endoparasitic fauna, making an assessment of their potential to act

as hosts/vectors for human pathogenic parasites problematic. Understanding parasite diversity within this context is therefore essential, both from a human health and conservation perspective. The ways in which anthroponotic impacts alter wildlife-pathogen dynamics, particularly within urban landscapes clearly need to be explored and the underlying pathways facilitating zooanthroponotic pathogen transmission at the human/wildlife interface need to be investigated.

5. Conclusion

This study reports the identification of four novel *Cryptosporidium* genotypes (bat genotype V-VIII) in wild and captive Grey-headed Australian flying foxes (NSW). It also provides the first account of zooanthroponotic pathogen transmission of *C. hominis* in flying foxes within a captive environment (QLD). This finding clearly highlights the potential for pathogen spillover at the human/flying fox interface within an Australian context. Valuable baseline data in relation to *Cryptosporidium* diversity and prevalence are provided, thereby addressing the current knowledge gap in relation to the endoparasitic fauna of Australian flying foxes.

Recent advances in sequencing techniques have considerably improved our understanding of *Cryptosporidium* taxonomy and transmission cycles (Ryan et al., 2014). Differential diagnostics not only enables the reliable identification of outbreak sources, but also informs treatment advice and underpins targeted control and management of zoonotic disease outbreaks (Chalmers and Katzer, 2013). Improvements in molecular tools coupled with accumulation in data are clearly vital in improving our understanding of the epidemiology of *Cryptosporidium* spp. and the underlying associations between humans and animals (Ryan et al., 2014).

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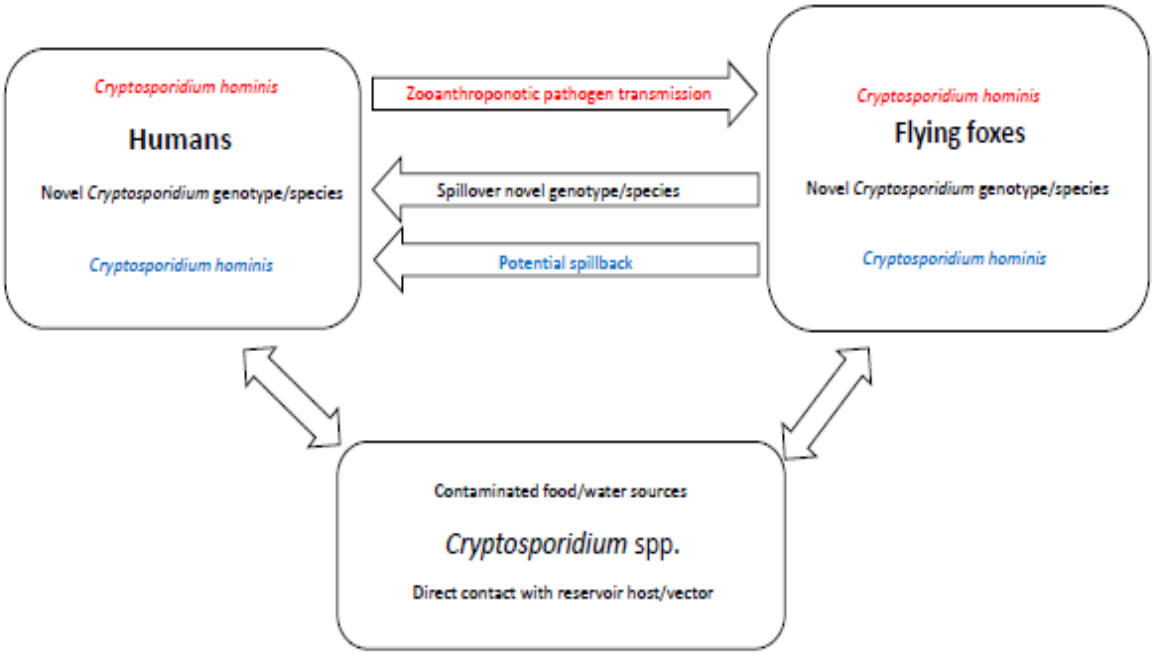
5.1 Highlights

- Evidence for zooanthroponotic pathogen transmission in Australian flying foxes
- *C. hominis* identified in captive flying foxes (*Pteropus conspicullatus*)
- Novel genotype/species identified in Australian flying foxes

5.2 Graphical abstract



Supplementary 1. PCR primers and corresponding primer sequences

Primer	Gene target	Direction	Amplicon length	Sequence 5' – 3'	Reference
LXF1	18s rRNA	Forward	1,325 bp	TTCTAGAGCTAATACATGCG	(Xiao et al., 1999a)
LXR1		Reverse		CCCATTTCCAAACAGGA	(Xiao et al., 2000)
18s IF	18s rRNA	Forward	298 bp	AGTGACAAGAAATAACAATACAGG	(Morgan et al., 1997b)
18s IR		Reverse		CCTGCTTTAAGCACTCTAATTTTC	
LXF2	18s rRNA	Forward	819 -825 bp	GGAAGGGTTGTATTTATTAGATAAAG	(Xiao et al., 1999a)
LXR2		Reverse		AAGGAGTAAGGAACAACCTCCA	
Act F1	Actin	Forward	~1095 bp	ATGRGWGAAGAAGWARYWCAAGC	(Sulaiman et al., 2002)
Act R1		Reverse		AGAARCAYTTTCTGTGKACAAT	
Act F2	Actin	Forward	~1066 bp	CAAGCWTTRGTTGTTGAYAA	(Sulaiman et al., 2002)
Act R2		Reverse		TTTCTGTGKACAAATWSWTGG	
AII F1	Actin	Forward	~830 bp	ATGCCVGGWRTWATGGTDGGTATG	(Ng et al., 2006)
Act6R		Reverse		GGDGCAACRACYTTRATCTTC	
All F2	Actin	Forward	~818 bp	GAYGARGCHCARTCVAARAGRGGTAT	(Ng et al., 2006)
All R1		Reverse		TTDATYTTCATDGTHGAHGGWGC	
OutF	*GP60*	Forward	~1442 bp	ACCACATTTTACCCACACATC	(Power et al., 2009)
OutR		Reverse		TCCTCACTCGATCTAGCTCA	
AtgF	*GP60*	Forward	~1000 bp	ATGAGATTGTCGCTCATTATCG	(Waldron et al., 2009)
Redundant stopR		Reverse		TTACAACACGAATAAGGCTGC	
M13F	Cloning	Forward		GTAAAACGACGGCCAG	
M13R		Reverse		CAGGAAACAGCTATGAC	

6 Appendix

Journal Instructions

INTERNATIONAL JOURNAL FOR PARASITOLOGY: PARASITES AND WILDLIFE

GUIDE FOR AUTHORS

INTRODUCTION

The *International Journal for Parasitology: Parasites and Wildlife (IJP:PAW)* publishes the results of original research on parasites of all wildlife, invertebrate and vertebrate. This includes free-ranging, wild populations, as well as captive wildlife, semi-domesticated species (e.g. reindeer) and farmed populations of recently domesticated or wild-captured species (e.g. cultured fishes). Articles on all aspects of wildlife parasitology are welcomed including taxonomy, biodiversity and distribution, ecology and epidemiology, population biology and host-parasite relationships. The impact of parasites on the health and conservation of wildlife is seen as an important area covered by the Journal especially the potential role of environmental factors, for example climate. Also important to the journal is 'one health' and the nature of interactions between wildlife, people and domestic animals, including disease emergence and zoonoses.

Types of articles

The principal form of publication is the full-length article which contains substantial, original research. The journal accepts brief reports that have similar subject scope as the full-length article, but do not merit a full-length publication. In addition, the journal commissions articles with emphasis on shorter, focused reviews of topical and emerging issues as well as strategically important subjects. The journal encourages critical comment and debate on matters of current controversy in the area of parasites and wildlife via "Short Communication".

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also http://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at: http://help.elsevier.com/app/answers/detail/a_id/286/p/7923.

Order of files

Manuscript should contain (in order) Title, Authors and addresses, Corresponding Author and address, Abstract, Keywords. In numbered sections: 1. Introduction; 2. Materials and methods; 3. Results; 4. Discussion; then Acknowledgements; References; Legends to Figures. Tables with their legends (in separate or combined files, numbered, in order). Figures (in separate files); preferred formats: JPEG, EPS or PDF. Supplementary and multimedia files.

Format

The preferred format for the text is Microsoft Word. The title page, abstract and text should be formatted with line numbers. The manuscript should be formatted to A4 size paper, in English, double spaced and with 2 cm margins.

PREPARATION

The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication. Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise. For brief reports, the Results and Discussion sections need to be combined.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself. The maximum length of the abstract is 300 words.
Additional comment: A 200 word limit was imposed by the Masters of Research committee.

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system.

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Immediately after the abstract, provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

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Avoid the use of abbreviations, but if necessary, authors should use the list (click here to see list) as a guide to those terms that need not be given in full, or define each abbreviation on first use.

Acknowledgments

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Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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Footnotes should only be used in tables. Indicate each footnote in a table with a superscript lowercase letter.

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Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

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Correct references are the responsibility of the author. Please ensure that all references cited in the text are included in the reference list.

References in the text start with the name of the author(s), followed by the publication date in brackets, e.g. 'Combes (2001) has shown the importance of ...', or '... has been described (Combes, 2001; Kumar et al., 2004) ...', using date order. More than one paper from the same author in the same year must be identified by the letters a, b, c, etc., placed after the year of publication. In the text, when referring to a work by two authors, use (Sangster and Dobson, 2002) or for more than two authors, the name of the first author should be given followed by et al. The references in the reference list should be in alphabetical order. References to journal articles should contain names and initials of all author(s), year of publication, article title, abbreviation of the name of the journal, volume number and page numbers. Unpublished data, personal communications and papers ' in preparation' or ' submitted' , abstracts (whether published or not) and these should not be listed in the references (but may be incorporated at the appropriate place in the text); work "in press" may be listed only if it has been accepted for publication. Personal communications must be accompanied by a letter or e-mail from the named person(s) giving permission to quote such information. References to books should also include the title (of series and volume), initials and names of the editor(s) and publisher and place of publication.

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Combes, C., 2001. Parasitism. The ecology and evolution of intimate interactions. University of Chicago Press, Chicago and London.

Kumar, N., Cha, G., Pineda, F., Maciel, J., Haddad, D., Bhattacharyya, M.K., Nagayasu, E., 2004. Molecular complexity of sexual development and gene regulation in *Plasmodium falciparum*. Int. J. Parasitol. 34, 1451-1458.

Pettersson, E.U., Ljunggren, E.L., Morrison, D.A., Mattsson, J.G., in press. Functional analysis and localisation of a delta-class glutathione S-transferase from *Sarcoptes scabiei*. Int. J. Parasitol.
Sangster, N.C., Dobson, R.J., 2002. Anthelmintic resistance. In: Lee, D.L. (Ed.), The biology of nematodes. Taylor and Francis, London and New York, pp. 531-567.

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

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As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references to published articles can be included in the reference list. Other web references such as software programs, databases and individual web pages, should have the reference details included at the appropriate place within the text.