A COMPARATIVE STUDY OF PARASITES IN CAPTIVE AND WILD TASMANIAN DEVILS

By

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DECLARATION

This thesis is written in the form of three journal articles from *The International Journal for Parasitology: Parasites and Wildlife*. The first article is a review article, while the second and third articles are original research papers.

I wish to acknowledge the following assistance in the research detailed in this report:

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

Liana Fay Wait

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OUTLINE OF CHAPTERS

Chapter 1. Review: Parasites of the Tasmanian devil and the potential impact of conservation management

Chapter 1 consists of a literature review of Tasmanian devil parasites and the Save the Tasmanian Devil Program. The review outlines current and historical knowledge of parasites in the Tasmanian devil, and discusses ways in which conservation management may theoretically change host-parasite interactions, both generally and with detail specific to the Save the Tasmanian Devil Program.

I reviewed the literature and wrote this chapter with feedback provided by Michelle Power, my supervisor, and Samantha Fox, our collaborator with the Save the Tasmanian Devil Program.

Chapter 2. Molecular characterization of *Cryptosporidium* and *Giardia* from Tasmanian devils

Chapter 2 describes the molecular characterisation of *Cryptosporidium* and *Giardia* from captive and wild Tasmanian devils. Faecal samples were collected from wild and captive devils, DNA was extracted, PCRs targeting *Cryptosporidium* and *Giardia* were performed, and sequence analysis of PCR amplicons allowed parasite species identification. The findings are discussed in the context of current knowledge of *Cryptosporidium* and *Giardia* in other Australian marsupials.

Faecal samples were collected by staff of the Save the Tasmanian Devil Program, Devil Ark, Healesville Sanctuary, Monarto Zoo, Taronga Zoo, and Western Plains Zoo. I participated in fieldwork and sample collection with the Save the Tasmanian Devil Program, performed all laboratory work, data analysis and statistics, and wrote the chapter with feedback from my supervisor, Michelle Power.

Chapter 3. Parasite diversity in the Tasmanian devil – a comparative study of wild and captive devils

Chapter 3 investigates gastrointestinal parasite prevalence and diversity in the Tasmanian devil, and compares findings between wild and captive populations. Zinc sulfate faecal flotations and microscopy were used to identify gastrointestinal helminths and coccidia, and prevalence data on *Cryptosporidium* and *Giardia* from Chapter 2 were also analysed.

Faecal samples were collected by staff of the Save the Tasmanian Devil Program, Devil Ark, Healesville Sanctuary, Monarto Zoo, Taronga Zoo, and Western Plains Zoo. I participated in fieldwork and sample collection with the Save the Tasmanian Devil Program, performed all laboratory work, data analysis and statistics, and wrote the chapter with feedback from my supervisor, Michelle Power.

CHAPTER 1

REVIEW: PARASITES OF THE TASMANIAN DEVIL AND THE POTENTIAL IMPACT OF CONSERVATION MANAGEMENT

Abstract

Threatened by devil facial tumour disease, the Tasmanian devil (*Sarcophilus harrisii*) is the focus of the Save the Tasmanian Devil Program. Conservation management may impact upon parasite ecology and present increased disease risk through stress and impaired immunity, and by exposing hosts to parasites to which they are immunologically naïve. It is becoming increasingly acknowledged that parasites perform important ecosystem functions, and it has been argued from a biodiversity perspective that parasites should be conserved in their own right. This review describes the Tasmanian devil, its disease and conservation, our current knowledge of Tasmanian devil parasites, and the potential for conservation-driven changes to the devil's parasite community. The limitations of our knowledge regarding Tasmanian devil parasites are highlighted, and the review closes with a recommendation that an evaluation be undertaken of the parasites of captive and wild devils; such an evaluation would to examine the possibility that conservation management may be changing the Tasmanian devil parasite community.

Keywords: Tasmanian devil, parasite ecology, conservation management, review

1. Introduction

It is becoming increasingly acknowledged that parasites play important roles in ecological communities (Hudson et al., 2006). As well as causing overt disease, parasites can indirectly affect their hosts by changing behaviour, reducing fecundity and growth, and increasing risk of predation (Marcogliese, 2004). These changes may effectively reduce the fitness of infected individuals relative to uninfected conspecifics and sympatric competitors (Marcogliese, 2004). Even subclinical disease may seriously impact a species' viability when interacting with other negative drivers such as reduced habitat, climate change, environmental pollution and competition from invasive species (Smith et al., 2009). There are many arguments supporting the conservation of parasitic species in their own right; parasites and other infectious agents are key ecological and evolutionary drivers; parasites are essential for the effective development of host immune systems; and, from an anthropocentric view, parasites may have research or medicinal purposes for humans (Gómez and Nichols, 2013; Spencer and Zuk, 2016). Additionally, parasite community ecology suggests that the loss of one parasite may alter disease risk for the host by changing competitive pressure on other parasites (Gómez and Nichols, 2013). Given the risks of disease and the importance of parasite conservation for biodiversity (Dougherty et al., 2015), it is imperative that we understand the impact of conservation management on parasite prevalence and diversity.

The Tasmanian devil (*Sarcophilus harrisii;* Dasyuridae: Marsupalia) is the largest extant carnivorous marsupial. Though once prevalent across mainland Australia, Tasmanian devils are now restricted to the island of Tasmania (Brown, 2006). Devils can be found state-wide in Tasmania, though their habitat is becoming increasingly fragmented by urban and agricultural development (Jones et al., 2004). Long-term habitat fragmentation has resulted in genetically distinct populations of devils in eastern and north-western Tasmania (Jones et al., 2004). Tasmanian devils are currently threatened by devil facial tumour disease (DFTD), caused by an infectious and invariably fatal cancer (Bender et al., 2014a). In response to the threat of DFTD, a nation-wide conservation program known as the Save the Tasmanian Devil Program was established (DPIPWE, 2014b).

This review describes the Save the Tasmanian Devil Program and emphasizes aspects of the program with implications for host-parasite relationships. It then examines our current and historical knowledge of parasites of the Tasmanian devil, highlighting gaps in our knowledge. The review closes with a discussion of the ways in which conservation management can effect parasite ecology, and provide recommendations for a systematic comparison of parasite diversity in captive and wild Tasmanian devils.

2. Disease in the devil – Devil Facial Tumour Disease

Tasmanian devils are threatened by DFTD, a lethal transmissible tumour. DFTD was first discovered in 1996 and has since spread through more than 85% of the Tasmanian devil population (Bender et al., 2014a). The disease is invariably fatal, and infected animals die due to starvation as a result of the tumour obstructing the oral cavity, or due to disseminated disease following metastasis (Bender et al., 2014a).

DFTD is unusual in that it is the cancerous cells themselves that are transmitted via allograft (Pearse and Swift, 2006). This transmission is facilitated by the extremely low genetic diversity seen in Tasmanian devils (Brüniche-Olsen et al., 2014). Tumours from different populations of devils have been shown to have identical karyotypes, indicating that DFTD is likely to have arisen from mutations in a single original host (Pearse and Swift, 2006). Studies of gene expression in DFTD cells have indicated that they are likely to be of Schwann cell origin. Schwann cells are a type of cell in the peripheral nervous system, responsible for creating myelin sheathes around neurons and also involved in immune-modulation via antigen presentation (Grueber et al., 2015). This immune-modulatory role may have helped the tumour initially evade the devil's immune system (Grueber et al., 2015). DFTD evades recognition by the immune system by down-regulating the expression of major histocompatibility complex (MHC) class I molecules, which are responsible for the presentation of foreign antigens to cytotoxic T cells (Bender et al., 2014b; Siddle et al., 2013). Such downregulation of MHC molecules should elicit tumour destruction by natural killer (NK) cells, part of the innate immune system (Grueber et al., 2015). However, although Tasmanian devils have functional NK cells, these NK cells do not recognize DFTD; this is an area currently being investigated (Grueber et al., 2015).

Transmission of DFTD is thought to occur via direct contact with an infected animal, especially through biting behavior (Hamede et al., 2013). The disease appears to be frequency-dependent rather than density-dependent, which has implications for the survival of the species as frequency-dependent diseases do not need a threshold population density to persist, and hence can cause extinction (McCallum et al., 2007). However, after more than ten years of monitoring of wild devil populations, no local extinction has been seen and long term diseased populations typically tend to persist at very low levels of abundance (pers. comm. Samantha Fox). DFTD is seen almost exclusively in adult devils and the highest rates of transmission seem to be during the mating season (Jones et al., 2007), when adult devils deliver penetrative bites to the head (Hamede et al., 2008).

DFTD has resulted in changes in life-history, including precocial breeding, semalparous rather than iteroparous reproduction (Jones et al., 2008), and an increased bias towards female offspring

(Lachish et al., 2009). There is also evidence that DFTD has resulted in lower dispersal rates in female devils and increased inbreeding, though it has not yet decreased genetic diversity (Lachish et al., 2011).

3. Conservation management of the Tasmanian devil

The Save the Tasmanian Devil Program (STDP), established in 2003, is working to save Tasmanian devils as a viable species in the face of DFTD (DPIPWE, 2014b). This program initially worked to develop an understanding of DFTD and its impact on the Tasmanian devil. The program then moved towards establishing a captive insurance population. The program's focus now includes establishing disease free populations in the wild and managing diseased wild populations (DPIPWE, 2014b).

The aim of the insurance population is to maintain a genetically diverse population of Tasmanian devils in captivity which could be used to re-populate Tasmania in the event that devils become extinct in the wild (Lees and Andrew, 2012). More than thirty zoos and wildlife parks in Australia and overseas are involved in the captive program, and the various populations are managed as a metapopulation in order to maintain gene-flow (Lees and Andrew, 2012). The program aims to maintain the Tasmanian devil as a viable species by building an insurance population of DFTD-free devils that display wild behaviours and retain at least 95% of the devil's current genetic diversity for a period of 50 years (Lees and Andrew, 2012). Another program objective is to conserve commensal, symbiotic and parasitic organisms associated with Tasmanian devils (DPIW and ARAZPA, 2007). This is a laudable goal as parasites have been shown to be drivers of biodiversity, and so changing host-parasite relationships could have ecosystem-wide implications (Hudson et al., 2006). However, current knowledge of parasites associated with Tasmanian devils is limited. The Save the Tasmanian Devil Program has undertaken preliminary work on parasite prevalence as part of routine health management, but no systematic assessment of parasites in the Tasmanian devil has yet been undertaken (Beveridge and Spratt, 2015). As such, this remains an important area for investigation.

Tasmanian devils in the insurance population are housed in a range of intensive enclosures and free-range facilities in Tasmania and on mainland Australian (Lees and Andrew, 2012). The program has also begun to establish wild populations of devils on disease-free islands and peninsulas off of Tasmania (Lees and Andrew, 2012). So far, devils have been released onto Maria Island and the Forestier Peninsula (DPIPWE, 2014a, 2015). These wild populations are expected to provide multiple benefits, including allowing devils to retain wild behaviours and to interact with the ecosystem (Lees and Andrew, 2012). Devils in these populations will also be subject to drivers

of natural selection, possibly strengthening the genetic diversity of the species. It was recommended that these populations be founded with wild-caught animals in order to preserve their natural commensal, symbiotic and parasitic fauna. However, other restricting factors meant that the founder animals released on Maria Island and the Forestier Peninsula had all spent considerable time in captivity (DPIPWE, 2014a).

4. Parasites of the Tasmanian devil

Twenty-five parasites representing broad taxonomic classes have been described in the Tasmanian devil; seven species of nematode, nine species of platyhelminth, three protozoa, and six ectoparasites (Tables 1 - 4). Most reports of parasites in the Tasmanian devil have been descriptive in nature, describing the morphology of the parasite and its location in or on the host. For many parasites found in devils, the complete life-cycles are unknown. Additionally, data on prevalence has only been provided for five of these parasites; *Trichinella pseudospiralis, Woolleya sarcophili, Anoplotaenia dasyuri, Toxoplasma gondii* and *Giardia spp.* (Davies, 1995; Gregory et al., 1975; HUMPHERY-SMITH and DURETTE-DESSET, 1981; Kettlewell et al., 1998; Obendorf et al., 1990). Much of this information is outdated as many of these parasites have not been reported in recent years; only 3/25 (12%) recorded Tasmanian devil parasites have been reported since 2000, and 21/25 (84%) have not been reported in the last 20 years.

4.1. Nematodes

Nematodes or "roundworms" constitute a diverse animal phylum that includes both free-living and parasitic species. Species of parasitic nematodes have diverse lifecycles and infect vertebrate and invertebrate hosts. Parasitic nematode lifecycles vary – some species have complex lifecycles involving multiple hosts, others have direct lifecycles involving a single host (Anderson, 2000). Likewise, within a host, classes of parasitic nematode occupy various niches including the gastrointestinal tract, the lymph or blood vessels of the host, or the host's muscle tissue (Anderson, 2000).

An important nematode of devils, *Baylisascaris tasmaniensis* is the only member of the genus *Baylisascaris* with a marsupial host, and the only ascarid found in marsupialsTasmanian (Sprent, 1970). Tasmanian devils, eastern quolls (*Dasyurus viverrinus*) , and spotted-tailed quolls (*D. maculatus*) act as definitive hosts, while common wombats (*Vombatus ursinus*) and brush-tailed possums (*Trichosurus vulpeca*) are amongst the known intermediate hosts (Obendorf, 1993). *Baylisascaris procyonis,* a closely related ascarid of raccoons (*Procyon lotor*) in the northern hemisphere, causes often-fatal larval migrans disease in humans (Sorvillo et al., 2002). There has

been speculation as to whether *B. tasmaniensis* could also cause larval migrans disease, but there have been no reports thus far (Bradbury, 2015).

Tasmanian devils are one of the main hosts in the sylvatic cycle of *Trichinella pseudospiralis*. The prevalence of *T. pseudospiralis* infection in Tasmanian devils was estimated as 70% (*n* =17) in 1990 (Obendorf et al., 1990). *Trichinella* species are maintained through a predator-prey life cycle, and Tasmanian devils are probably infected through predation, carrion feeding, and cannibalism (Obendorf et al., 1990). Infections with *T. pseudospiralis* have also been identified in eastern quolls (*D. viverrinus)*, spotted-tail quolls (*D. maculatus*), brush-tailed possums (*T. vulpeca)*, masked owls (*Tyto novaehollandiae*), and marsh harriers (*Circus aeruginosus*) (Obendorf et al., 1990; Obendorf and Clarke, 1992). *T. pseudospiralis* can cause zoonotic infections, and one human case has been documented in Tasmania (Andrews et al., 1994).

The nematode *Woolleya sarcophili* is found in the small intestines of Tasmanian devils. The most recent published information on this parasite was in 1981, when its taxonomy was revised from *Nicollina sarcophili* to *W. sarcophili* (HUMPHERY-SMITH and DURETTE-DESSET, 1981). In this revisory paper, 2/25 dissected devils were infected – one with three female worms and the other by a single female worm (HUMPHERY-SMITH and DURETTE-DESSET, 1981). The morphological features of male *W. sarcophili*, specifically the spicules, have not been described (HUMPHERY-SMITH and DURETTE-DESSET, 1981). The eggs of *W. sarcophili* have been described as being "of the typical trichostrongyle type" and measure 70 x 40 μm (Cameron, 1931).

Angiostrongylus cantonensis, the "rat lungworm", is a strongyloid nematode that is thought to have been introduced to Australia along with its rat definitive host (Prociv and Carlisle, 2001). This nematode has a complex lifecycle involving a molluscan intermediate host (Prociv and Carlisle, 2001). *A. cantonensis* aberrantly infects a broad range of mammals, including marsupials, and has been reported once in a captive Tasmanian devil (Munday, 1988). These aberrant infections can cause severe pathology; in humans, *A. cantonensis* causes sometimes fatal eosinophilic meningoencephalitis (Prociv and Carlisle, 2001).

Tasmanian devils serve as the definitive host for *Physaloptera sarcophili* along with northern quolls (*Dasyurus hallucatus*), another species of dasyurid (Johnston and Mawson, 1940). Adult and larval stages are found in the stomach. The complete life cycle of this species is not known, but other members of *Physaloptera* have life cycles involving insect intermediate hosts and can also be transmitted through the ingestion of paratenic hosts (Olsen, 1980).

Cercopithifilaria johnstoni is a microfilarial nematode that infects the subcutaneous tissues of Tasmanian devils and a range of other Australian marsupials and murids. Ixodid ticks act as the

intermediate host, with larval development of *C. johnstoni* only occurring while a tick is off of a host, and infectious third stage larvae are transmitted when infected ticks feed (Spratt and Haycock, 1988).

The nematode *Cyathospirura seuratis* is found in the stomachs of Tasmanian devils and other dasyurids as well as eutherian carnivores including cats and foxes (Beveridge and Spratt, 2003). These reddish nematodes are typically found free in the stomach lumen, though related species can be located in stomach nodules. In Tasmanian devils, these nematodes were initially described as a new species, *Cyathospirura dasyuridis* (Mawson, 1968), but it was later synonymized with the previously described *C. seuratis* (Hasegawa et al., 1993). The life cycles of *Cyathospirura* species have not been elucidated but are presumed to involve intermediate and paratenic hosts upon which the carnivorous definitive hosts prey.

4.2. Platyhelminthes

Platyhelminthes or "flatworms" include parasitic species in three sub-classes, the monogenea, digenea and cestodes. Of these three classes, only digenea and cestodes are known to parasitise Tasmanian devils. The digenea (Platyhelminthes: Trematoda), also known as trematodes or flukes, represent arguably the largest group of metazoan endoparasites (Olson et al., 2003). Digenea have complex lifecycles involving three distinct life stages and between one and four hosts (Cribb et al., 2003). Cestodes (Platyhelminths: Cestoda) are a diverse group of metazoan endoparasites, known colloquially as tapeworms (Olson et al., 2001). The Cylcophillidea, the group of cestodes that parasitise mammals and other tetrapods, have complex life-cycles involving different life-stages in multiple host-species (Mackiewicz, 1988). These lifecycles frequently rely upon predator-prey interactions for transmission (Mackiewicz, 1988).

4.2.1. Digenea

Three digeneans have been described in the Tasmanian devil, *Mehlisia acuminata*, *Neodiplostomum sarcophili* and *Neodiplostomum diaboli*. None of these species has a fully elucidated life cycle, though most digeneans have complex life cycles as described above.

Mehlisia acuminata is a fasciolid trematode found in the intestines of Tasmanian devils and eastern quolls (*D. viverrinus*). The type specimen was described from an eastern quoll in 1913 (Johnston, 1912), and this species has not been mentioned in the primary literature since. Eggs measure approximately 134 x 79 µm and are light yellow in colour (Johnston, 1912). *Neodiplostomum sarcophili* was described in 1957 under the name *Fibricola sarcophila* but its taxonomy was revised in 1993. (Cribb and Pearson, 1993). Adult flukes range in length from 1.15 – 1.85 mm and are found in the intestine. Tasmanian devils are the only known definitive host. Eggs

measure approximately 98 - 112 x 70 µm (Sandars, 1957). Tasmanian devils and eastern quolls (*D. viverrinus*) serve as the definitive host for *Neodiplostomum diabolic* (Cribb and Pearson, 1993). This species is distinguished from others in the genus *Neodiplostomum* based on its trilobate shaped testis. Adults range between 1.95 – 2.05 mm in length and are presumed to be located in the intestine of the definitive host, though their site within the host has never been formally recorded. Eggs are approximately 75 x 125 µm (Dubois and Angel, 1972).

4.2.2. *Cestodes*

Anoplotaenia dasyuri is a very common and apparently non-pathogenic cestode of Tasmanian devils. A 1975 survey found adult worms in 293/294 devils, with worm burdens of over 1000 in most adult devils (Gregory et al., 1975). *A. dasyuri* follows a predator-prey life cycle, with adult cestodes occurring in the small intestine of the definitive host, and larval stages (metacestodes) occurring in the tissues of intermediate hosts. Spotted-tailed quolls (*D. maculatus*) have also been shown to act as definitive hosts for *A. dasyuri*, and eastern quolls (*D. viverrinus*) are a putative definitive host (Gregory et al., 1975). Adult cestodes have also been isolated from feral cats and rural dogs, but these were poorly developed for the most part, and it has been suggested that the gastrointestinal tracts of placental carnivores may be unsuitable for full maturation of *A. dasyure* (Gregory et al., 1975). Several species of macropod (kangaroos and wallabies) have been identified as natural intermediate hosts, and particularly high burdens of metacestodes have been isolated from Bennett's wallabies (*Macropus rufogriseus rufogriseus*) (Gregory et al., 1975). Brush-tailed possums (*T. vulpecula)* and laboratory rodents have also been successfully experimentally infected (Beveridge et al., 1975). Bennett's wallabies and brush-tailed possums are known to be common sources of food for devils (Pemberton et al., 2008).

Dasyurotaenia robusta, a cestode found in the small intestine of Tasmanian devils, (Beveridge, 1984) is classified as Rare under the Tasmanian Threatened Species Protection Act, 1995. This cestode has been reported in captive animals, but in the wild it appears to be restricted to one location – Collins Gap (Beveridge and Spratt, 2015). *D. robusta* was originally described from specimens obtained from captive animals (Beveridge, 1984), and its life cycle has not been elucidated. Members of Dasyurotaenia are distinguished from morphologically similar cestodes by a large scolex that embeds deeply into the intestinal mucosa of the host (Beveridge, 1984).

There are no morphological guidelines for the differentiation of *A. dasyuri* and *D. robusta* eggs. However, *A. dasyuri* eggs can be differentiated from those of *Taenia spp*. by their ellipsoid rather than spherical shape, and by the irregular shape of their embyophoric blocks compared with the

rhomboidal embyrophoric blocks of *Taenia spp*. when viewed under immersion oil (pers. comm. Ian Beveridge).

The taxonomic grouping of *A. dasyuri* and *D. robusta* is uncertain and a cause of controversy. These cestodes were initially placed with the Taeniids, a related group of eutherian cestodes, based on morphological similarities, making them the only Taeniids of Australian carnivorous marsupials. However, there is speculation about the appropriateness of this grouping given Australia's extended isolation from eutherian mammals and their cestodes (Beveridge and Jones, 2002; Beveridge et al., 1975). Though the Taeniids have had a molecular phylogeny mapped (Nakao et al., 2013), but attempts to map the phylogenies of *Dasyurotaenia* and *Anoplotaenia* have thus far relied solely on morphological features and ontology (Hoberg, 1999). Modern molecular techniques could shed light on the issue of where to place *Dasyurotaenia* and *Anoplotaenia*.

Three cestode species with canid definitive hosts, *Taenia pisiformis, Taenia hydatigena* and *Taenia ovis*, have been identified in the Tasmanian devil but are not parasites of the devil per se. The only mention of *T. pisiformis* in Tasmanian devils is the record that a specimen of this species from a Tasmanian devil can be found in the Australian Helminthological Collection at the South Australian Museum (Spratt et al., 1991). Tasmanian devils have been successfully experimentally infected with both *T. hydatigena* (Gregory, 1976) and *T. ovis* (Gregory, 1972). These experimental infections aimed to determine whether devils could be acting as a source of transmission for these parasites, but no natural infections have been reported for either species (Gregory, 1976). Devils have also been experimentally infected with *Echinococcus granulosis*, but no infections were established (Gregory, 1976).

Tasmanian devils are an accidental intermediate host for *Spirometra erinacei*, a cosmopolitan cestode that is assumed to have been introduced to Australia along with its dog, cat and fox definitive hosts (Beveridge and Spratt, 2003). This cestode has a complex life cycle involving a free living stage (coracidium), a primary copepod intermediate host, various secondary intermediate hosts which harbour plerocercoid larve in spargana, and the final definitive host, a carnivorous mammal (Lee et al., 1990).

4.3. Protozoa

The protozoa are a diverse group of single-celled, eukaryotic organisms, some of which hold parasitic life-styles. Parasitic protozoa are phylogenetically diverse and have varied life-histories, sometimes involving free-living stages and complex lifecycles (Vickerman, 1992).

Our current knowledge of protozoal parasites in the Tasmanian devil is particularly limited, and the available information is somewhat outdated. Almost all studies published on protozoa in the

Tasmanian devil were conducted prior to the advent of molecular techniques that allow for the identification of these organisms down to a species level. As a result, several protozoan parasites found in Tasmanian Devils have not been identified or classified beyond the genus level (Davies, 1995; Kettlewell et al., 1998; Munday et al., 1978). As well as enabling species identification, molecular techniques have hugely increased our knowledge of the genetic diversity, host specificity and taxonomy of many protozoal genera (Cacciò and Ryan, 2008; Xiao and Ryan, 2004).

Giardia is a genus of flagellated protozoan parasites that causes gastrointestinal infections in a broad range of vertebrate host species, including mammals, birds, and amphibians (Appelbee et al., 2005). Two studies have reported on the prevalence of *Giardia* in Tasmanian devils with the first estimate of 8.33% (n = 12) (Davies, 1995) and the second 6% (n = 32) (Kettlewell et al., 1998). Both of these studies relied purely on microscopy and hence were only able to identify *Giardia* to the genus level, as species of *Giardia* are morphologically indistinguishable. A review of *Giardia* in mammalian wildlife reported on the unpublished finding of a novel *Giardia* genotype from a Tasmanian devil, however they did not specify the origin of the devil or the gene targeted (Appelbee et al., 2005). Estimates of the prevalence of *Giardia* in other species of Australian marsupials range from 1.3% to 13.8% (Adams et al., 2004; Ng et al., 2011; Thompson et al., 2010; Vermeulen et al., 2015). No significant differences in the prevalence of *Giardia* have been found between captive and wild populations of marsupials (Thompson et al., 2008; Vermeulen et al., 2015).

Toxoplasma gondii is a ubiquitous protozoan pathogen capable of infecting most if not all warm-blooded animals (Tenter et al., 2000). In Tasmanian devils, the seroprevalence of *T. gondii* has been estimated as 33% (n = 18) (Hollings et al., 2013). This is interesting, as clinical toxoplasmosis has not been described in devils, though it does cause debilitating disease in other dasyurid marsupials (Obendorf, 1993). It should be noted, however, that the study of *T. gondii* in devils looked at seroprevalence only, which provides an indication of exposure to *T. gondii* but not infection.

Sarcocystis is a genus of cyst-forming protozoan parasite (Tenter, 1995). A 1978 study found *Sarcocystis*-like cysts in the skeletal muscle tissue of a single Tasmanian devil, and in a range of other marsupial and eutherian mammals in Tasmania and on mainland Australia (Munday et al., 1978). Sporulated sporocysts, the parasitic stages resulting from *Sarcocystis* sexual reproduction, were also found in scrapings of the intestinal mucosa of a single Tasmanian devil, suggesting that Tasmanian devils could act as the definitive host for this species of *Sarcocystis* (Munday et al., 1978).

In addition to the published information on protozoa in Tasmanian devils (Table 3), routine faecal flotations of wild Tasmanian devils have detected *Eimeria* oocysts (pers.comm. Sarah Peck). *Eimeria* species are generally very host-specific and most species parasitise herbivores. However, *Eimeria* oocysts sometimes occur as pseudoparasites in the faeces of carnivores as a result of ingestion of oocysts through predation or coprophagy (Gressler et al., 2009). Heavy, subclinical infections with *Eimeria* are common in many marsupials, including species upon which the Tasmanian devil preys (Barker et al., 1989). Thus, it remains to be determined whether *Eimeria* oocysts found in Tasmanian devil faeces are indicative of a true infection, or if they are pseudoparasites.

4.4. Ectoparasites

Mammalian ectoparasites comprise multiple taxa of arthropods including ticks and mites (Arachnida: Acari), fleas and lice (Insecta: Pterygota), and some dipteran species (Insecta: Panorpida) (Lehmann, 1993). To classify as an ectoparasite, an arthropod must be associated with a host for most, but not necessarily all, of its lifetime (Nelson et al., 1975).

Two species of mite have been found to infect the Tasmanian devil - *Diabolicoptes sarcophilus* and *Satanicoptes armatus.* Male and female specimens of *D. sarcophilus* were extracted from the faeces of a single Tasmanian devil, but it is presumed to be a skin parasite (Fain and Domrow, 1974). The second mite, *Satanicoptes armatus,* caused sarcoptic mange in a single captive Tasmanian devil in London (Fain and Laurence, 1975). Neither of these mites has been mentioned in the primary literature since they were first described in 1974 and 1975, respectively.

Three tick species have been reported from Tasmanian devils – *Ixodes holocyculus, Ixodes tasmani* and *Ixodes fecialis.* All three of these species parasitise a wide range of marsupial and eutherian host species in addition to Tasmanian devils (Roberts, 1960). *I. holocyclus*, along with other ixodid ticks, can act as a vector for the nematode *C. johnstoni* (Spratt and Haycock, 1988), and is also important as the cause of "tick paralysis" in naïve domestic animals and humans, though native marsupials are generally immune to this effect (Stone et al., 1989). Tasmanian devil ticks have also been examined for their role as possible vectors of disease. *I. tasmani* and *I. holocyclus* ticks were pooled from wild and captive devils into 44 samples according to host, and the results found a spotted-fever group *Rickettsia* in 45.5% and a *Hepatozoon spp*. in 34.1% of pooled samples (Vilcins et al., 2009). The authors of this study suggested that this finding pointed to two new Tasmanian devil pathogens (Vilcins et al., 2009). However, *Rickettsia* and *Hepatozoon spp*. were detected only in ticks and not in Tasmanian devils themselves, and there have been no reported manifestations of rickettsial disease or hepatozoonosis described in devils.

The flea *Uropsylla tasmanica* is the only flea known to have an endoparasitic phase within its life cycle (Williams, 1991). The life cycle of *U. tasmanica* involves four larval stages, the first three being endoparasitic or occurring within the tissues of the host rather than on the surface. The fourth stage larvae leave the host and build cocoons in leaf litter, from which they emerge as adults in response to mechanical stimulation (Pearse, 1981). As well as parasitising Tasmanian devils, this flea is found on eastern quolls (*D. viverrinus)*, spotted-tail quolls (*D. maculatus)* and western quolls (*D. geoffroii)*. There are no primary references to *U. tasmanica* in Tasmanian devils, though unpublished observations of this flea in Tasmanian devils have been referenced, and these fleas have also been occasionally observed in wild devils during routine monitoring by the STDP (pers. comm. Samantha Fox).

Table 1

Parasitic nematodes of the Tasmanian devil.

Phylum	Order	Species	Type Location of Host	Prevalence	Last report [+]	References
Nematoda	Ascaridida	*Baylisascaris tasmaniensis*	Wild	NR	1970	Sprent (1970)
	Trichocephalida	*Trichinella pseudospiralis*	Wild	70.0% (*n* = 17)	1990	Obendorf et al. (1990)
	Rhabditida	*Woolleya sarcophili*	Captive and wild	8.0% (n = 25)	1981	Humphery-Smith and Durette-Desset, 1981; Cameron (1931)
	Strongylida	*Angiostrongylus cantonensis**	Captive	NR	1978 (pers. comm., cited 1988)	Munday (1988)
	Spirurida	*Physaloptera sarcophili*	Wild	NR	1940	Johnston and Mawson (1940)
		Cercopithifilaria johnstoni	Wild	NR	1975	Spratt and Varughese (1975)
		*Cyathospirura seurati**	Wild	NR	1968	Hasegawa et al. (1993); (Hasegawa et al., 1993); Mawson (1968)

NR = not recorded. [+] Refers to last record of each species in the primary literature. *Indicates parasitic species that are likely to have been introduced to Tasmanian devils when their eutherian hosts were introduced to Tasmania.

Table 2

Parasitic platyhelminthes of the Tasmanian devil

Phylum	Order	Species	Type Location of Host	Prevalence	Last report [+]	References
Platyhelminthes	Diplostomida	*Neodiplostomum sarcophili*	Wild	NR	1993	Sandars (1957)
		Neodiplostomum diaboli	Wild	NR	1972	Dubois and Angel (1972)
	Plagiorchiida	*Mehlisia acuminata*	Wild	NR	1912	Johnston (1912)
	Cyclophillidea	*Taenia pisiformis**	NR	NR	NR	Spratt et al. (1991)
		*Taenia hydatigena**	Experimental	NR	1976	Gregory (1976)
		*Taenia ovis**	Experimental	NR	1972	Gregory (1972)
		Anoplotaenia dasyuri	Wild	99.7% (*n* = 294)	1975	Gregory et al. (1975)
		Dasyurotaenia robusta	Captive and wild	NR	1990 (unpublished data, cited 2015)	Beveridge and Spratt (2015); (Beveridge, 1984; Beveridge and Spratt,
	Diphyllobothriidea	*Spirometra erinacei**	Wild	NR	1991	Spratt et al. (1991)

NR = not recorded. [+] Refers to last record of each species in the primary literature. *Indicates parasitic species that are likely to have been introduced to Tasmanian devils when their eutherian hosts were introduced to Tasmania.

Table 3

Parasitic protozoa of the Tasmanian devil.

Phylum	Order	Species	Type Location of Host	Prevalence	Last report [+]	References
Protozoa	Diplomonadida	*Giardia spp.*	Wild	8.33% (*n* = 12) 6.0% (*n* = 32)	1998	Kettlewell et al. (1998); Davies (1995); (Kettlewell et al., 1998)
	Eimeriida	*Sarcocystis spp.*	Wild	3.4% (n=29)[a] 2.0% (n=50)[b]	1978	Munday et al. (1978)
		*Toxoplasma gondii**	Wild	33.0% (*n* = 18)	2013	Hollings et al. (2013)

[+] Refers to last record of each species in the primary literature.*Indicates parasitic species that are likely to have been introduced to Tasmanian devils when their eutherian hosts were introduced to Tasmania. [a] Indicates prevalence of cysts stage in muscle tissue; [b] indicates prevalence of sporolated sporocysts in gastrointestinal scrapings.

Table 4

Ectoparasites of the Tasmanian devil

Phylum	Order	Species	Type Location of Host	Prevalence	Last report +	References
Arthropoda	Sarcoptiformes	*Satanicoptes armatus*	Captive (London)	NR	1975	Fain and Laurence (1975)
		Diabolicoptes sarcophilus	Wild	NR	1974	Fain and Domrow (1974)
	Acari	*Ixodes holocyclus*	Wild	NR	2009	Vilcins et al. (2009)
		Ixodes tasmani	Wild	NR	2009	Vilcins et al. (2009)
		Ixodes fecialis	Wild	NR	1964	Green and Ellis (1967)
	Siphonaptera	*Uropsylla tasmanica*	Wild	NR	1991 (reference to unpublished observations)	Williams (1991)

NR = not recorded. + Refers to last record of each species in the primary literature.

5. Conservation management and host-parasite interactions

Conservation management may alter host-parasite interactions in several ways. Keeping animals in captivity may result in increased disease risk by inducing stress and impaired immunity, and by exposing hosts to parasites to which they are immunologically naïve (Cunningham, 1996). Additionally, animals are often housed at higher densities than would naturally occur, resulting in increased parasite burdens. Where this is the case, animals may often be treated with anti-parasitic drugs to reduce parasite load. Such anthelmintic treatment previously formed part of the health management of the Tasmanian devil insurance population (Jones et al., 2007), though it is not a routine practice at present (pers. comm. Samantha Fox). Anti-parasitic treatment not only alters host-parasite relationships, but can also result in increased disease risk; if treated animals are released into the wild, at the individual-level they will not have had the opportunity to develop acquired immunity, and herd immunity will be reduced or lost (Lyles and Dobson, 1993). Rather than eliminating parasites in captive populations, it is recommended that animals be exposed to low levels to enable individuals to acquire immunity (Viggers et al., 1993), and to allow the maintenance of genetic and other adaptations within the population (Cunningham, 1996). Conversely, if animals harbour a specific parasite that does not occur at the release site, it is desirable to eliminate it prior to release (Cunningham, 1996; Woodford, 2000).

Animal translocation inevitably results in parasite translocation, with parasite transfer occurring in both directions – from the reintroduced animals to the extant population and vice versa (Viggers et al., 1993). The founding of a captive population can be considered a special type of translocation, and often involves mixing individuals from different origins. In the case of the Tasmanian devil insurance population, founding members originate from different regions of Tasmania, some of which are geographically isolated (Lees and Andrew, 2012). The north-western and eastern populations of Tasmanian devils are known to be genetically distinct (Jones et al., 2004), and so it is plausible that they could also have distinct endemic parasites. If this were the case, co-housing devils from different wild populations in captivity could result in the transfer of novel parasites to naïve individuals.

Captive devils could also be exposed to novel parasites through contact with humans and other captive species. Animal handling can result in the transmission of human-specific parasites, and, for animals housed in a zoo environment, parasites could be transferred mechanically by zookeepers from other captive species, or transmitted via contaminated food (Daszak et al., 2000). However, the STDP has made efforts to minimize these potential

transmission events by enacting a latex glove policy during all handling of STDP devils and their food (pers. comm. Samantha Fox). Nevertheless, if such a transmission event were to occur and an infected captive devil was released into the wild, these novel parasites would be released with them, with unknown consequences for extant devil populations. As a species, Tasmanian devils may be more susceptible to emerging disease threats due to low genetic diversity (Brüniche-Olsen et al., 2014). Inter-specific parasite transfer can also occur, particularly between related host species (Cunningham, 1996). This is of concern for Tasmanian devil reintroductions as devils share much of their habitat with the spotted-tailed (*D. maculatus*) and eastern quolls (*D. viverrinus*), two related species of dasyurid (Jones and Barmuta, 2000).

6. Concluding remarks and recommendations

Current knowledge of parasites in the Tasmanian devil is limited, and, though some parasite monitoring is performed as part of the Save the Tasmanian Devil Program, there has been no systematic evaluation of how conservation management may be changing parasite diversity and prevalence. I recommend that such an evaluation be undertaken. A systematic evaluation of Tasmanian devil parasites would provide multiple benefits. Such an assessment would (1) Generate baseline data on Tasmanian devil parasites; (2) Identify transfer of human parasites into devils that may be occurring as a result of devil management; and (3) Contribute to conserving parasites and symbionts associated with devils, a goal of the STDP. A parasite wide evaluation would allow for a risk analysis of emerging disease threats and the potential for spillover of pathogens from humans into Tasmanian devils, thereby bolstering Tasmanian devil conservation efforts.

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CHAPTER 2

MOLECULAR CHARACTERISATION OF *CRYPTOSPORIDIUM* AND *GIARDIA* FROM TASMANIAN DEVILS

Abstract

Cryptosporidium and *Giardia* are two ubiquitous waterborne genera of protozoan parasite that have previously been described in a range of Australian marsupials. This study investigated *Cryptosporidium* and *Giardia* in the Tasmanian devil, an endangered Australian marsupial. Tasmanian devils are currently threatened by devil facial tumour disease, a lethal transmissible cancer, and are the focus of the Save the Tasmanian Devil Program. This study used molecular techniques to investigate whether conservation management might be changing the prevalence and diversity of *Cryptosporidium* and *Giardia* between captive and wild populations of Tasmanian devils. A comparison of prevalence data between wild and captive populations showed that both *Cryptosporidium* and *Giardia* were significantly more prevalent in wild devils ($p < 0.05$); *Cryptosporidium* was identified in 37.9% of wild devils but only 10.7% of captive devils, while *Giardia* was identified in 24.1% of wild devils but only 0.82% of captive devils. Molecular analysis identified the presence of a novel genotype of *Cryptosporidium*, as well as samples with high sequence similarity to *C. fayeri*, *C. muris*, and *C. galli*. Two novel genotypes of *Giardia* were identified, and *G. duodenalis* BIV, a zoonotic genotype of *Giardia*, was also identified in a single captive Tasmanian devil. These findings suggest that conservation management may be changing host-parasite interactions in the Tasmanian devil, and the presence of *G. duodenalis* BIV in a captive devil points to possible human-devil pathogen transmission.

Key words: Cryptosporidium, Giardia, Tasmanian devil, conservation management

1. Introduction

Cryptosporidium and *Giardia* are two ubiquitous protozoan parasites capable of infecting a wide range of vertebrate species, including humans (Appelbee et al., 2005). There are multiple species and genotypes of *Cryptosporidium* and *Giardia*, and these have varying host-specificity and pathogenicity (Appelbee et al., 2005). Most species of *Cryptosporidium* and *Giardia* are morphologically indistinguishable, and so molecular tools have been instrumental in allowing for species identification and differentiation (Appelbee et al., 2005; Xiao, 2010). Both parasites are transmitted via direct contact with an infected host or through ingestion of contaminated food or water (Thompson, 2000; Xiao, 2010). *Cryptosporidium* and *Giardia* are of public health and agricultural significance as causes of enteric disease in humans and domestic animals (Cacciò and Ryan, 2008; Feng and Xiao, 2011; Xiao et al., 2004). Studies of both parasites in wildlife have generally focused on ascertaining whether wildlife hosts might act as disease reservoirs for humans or domestic animals (Appelbee et al., 2005). As such, little is known about the impact of *Cryptosporidium* and *Giardia* on wildlife species themselves. However, the ubiquity of *Cryptosporidium* and *Giardia*, along with the presence of species with both broad and narrow host-specificity within each genera, make them useful indicators for interactions between humans, domestic and wild animals.

Cryptosporidium has been identified in 16 marsupial species worldwide, including 14 Australian marsupials (Power, 2010). Two marsupial *Cryptosporidium* species, *C. fayeri* and *C. macropodum*, have been characterized from Australian marsupials, and there are multiple cryptic genotypes (Power, 2010). Estimates of the prevalence of *Cryptosporidium* in populations of Australian marsupials range from 6.7% to 12.2% (Hill et al., 2008; McCarthy et al., 2008; Power et al., 2005; Vermeulen et al., 2015a; Yang et al., 2011). Differences in prevalence have been noted within populations during different seasons (Power et al., 2005), and higher prevalence rates have been reported in populations from urbanised or agricultural settings (Hill et al., 2008; McCarthy et al., 2008). *Cryptosporidium* has been detected in both wild and captive marsupials (Power, 2010), but only one study has directly compared wild and captive populations, finding no significant difference in *Cryptosporidium* prevalence between captive and wild brush-tailed rock wallaby (*Petrogale pencillata*) populations (Vermeulen et al., 2015a).

Giardia has been identified in many species of Australian marsupial, and molecular studies have allowed identification of the exact species present in marsupials. *G. duodenalis* has been the species identified in all except one of these molecular studies (McCarthy et al., 2008; Thompson et al., 2008; Vermeulen et al., 2015b); the exception being the finding of a host-specific species of *Giardia*, named *G. peramelis*, in the quenda (Hillman et al., 2016). Estimates of prevalence of

Giardia in Australian marsupials range from 1.3% to 13.8% (Adams et al., 2004; Ng et al., 2011; Thompson et al., 2010; Vermeulen et al., 2015b). No significant differences in the prevalence of *Giardia* have been found between captive and wild marsupial populations (Thompson et al., 2008; Vermeulen et al., 2015b).

This study investigated the prevalence and diversity of *Cryptosporidium* and *Giardia* in an endangered Australian marsupial, the Tasmanian devil (*Sarcophilus harrisii*). The Tasmanian devil is the largest extant carnivorous marsupial. Though once prevalent across mainland Australia, Tasmanian devils have been restricted to the island of Tasmanian for the last 3000-4000 years (Brown, 2006). Devils can be found state-wide in Tasmania, though their habitat is becoming increasingly fragmented by urban and agricultural development (Jones et al., 2004). Tasmanian devils are currently threatened by a lethal transmissible tumour, devil facial tumour disease (DFTD) (Bender et al., 2014). DFTD was first discovered in 1996 and has since spread through more than 85% of the Tasmanian devil population (Bender et al., 2014). Due to the serious threat posed by DFTD, Tasmanian devils are subject to a conservation management program known as the Save the Tasmanian Devil Program (STDP) (DPIPWE, 2014). The primary goal of the program has been to establish a captive insurance population of genetically diverse and DFTD-free devils. Tasmanian devils in the insurance population are housed in a range of intensive enclosures and free-range facilities in Tasmania and on mainland Australia (Lees and Andrew, 2012). The recovery program has also begun to establish wild populations of devils on DFTD-free islands and peninsulas off of Tasmania (Lees and Andrew, 2012). These wild populations are expected to provide multiple benefits, including allowing devils to maintain wild behaviours and to interact with the natural ecosystem (Lees and Andrew, 2012). Establishment of DTFD-free wild populations also facilitates one of the conservation program's other goals: to conserve commensal, symbiotic and parasitic organisms associated with Tasmanian devils (DPIW and ARAZPA, 2007).

Tasmanian devils are known to host a variety of parasites, including nematodes, cestodes, digenea, ectoparasites and protozoa (Beveridge and Spratt, 2003). Two protozoal genera, *Giardia* and *Sarcocystis*, have been detected in Tasmanian devils, but studies of these parasites in devils have relied on classical techniques and have not been able to identify the parasites beyond a genus level (Davies, 1995; Kettlewell et al., 1998; Munday et al., 1978). Two such studies have reported on the prevalence of *Giardia* in Tasmanian devils with the first estimate of 8.3% (n = 12) (Davies, 1995) and the second 6.0% (n = 32) (Kettlewell et al., 1998). An unpublished report identified a novel genotype of *Giardia* from a Tasmanian devil (Appelbee et al., 2005), however there is not genetic data for this genotype on GenBank, nor is there information on the origin of the host. Antibodies specific to a third protozoan, *Toxoplasma gondii*, have been detected in the blood of

Tasmanian devils, but parasite stages themselves have not been isolated from devils (Hollings et al., 2013). *Cryptosporidium* has never been reported from Tasmanian devils.

The aims of this study were (1) to characterize *Cryptosporidium* and *Giardia* species in Tasmanian devils, and (2) to determine whether conservation management may be changing the prevalence and diversity of *Cryptosporidium* and *Giardia* in Tasmanian devils. To achieve this, molecular typing was used to characterize *Cryptosporidium* and *Giardia* from three different Tasmanian devil population types with varying levels of human contact: intensively managed captive populations, free-range captive populations, and wild populations of devils. Additionally, pre- and post-release faecal samples were analysed from a group of captive devils that were released into the wild in order to examine potential changes to the parasite community as a result of re-wilding.

2. Materials and methods

2.1. Sample collection

A total of 190 faecal samples were collected from 167 Tasmanian devils (Table 1). The sample populations consisted of five intensively managed captive populations (Monarto Zoo, Healesville Sanctuary, Taronga Zoo, Western Plains Zoo, and the Cressy STDP breeding facility), three free-range captive populations (Devil Ark and the STDP free-range enclosures at Bridport and on the Freycinet Peninsula), and two wild populations (Table Mountain and Narawntapu National Park). All samples were collected between July 2015 and February 2016, with the exception of 18 samples from Devil Ark that were collected in June 2014. Wild samples were collected from July 2015 – February 2016, free-range captive samples were collected from November 2015 – February 2016, and intensive captive samples were collected from October 2015 – February 2016. Additionally, pre- and post-release faecal samples were collected from 16 devils that were vaccinated with a trial vaccine against DFTD and released into Narawntapu National Park in September 2015 (DPIPWE, 2015). The vaccine-trial devils were bred in captivity as part of the insurance population and were housed in the Freycinet and TasZoo free-range captive enclosures for 4 – 6 months prior to release (pers. comm. Samantha Fox). Pre-release samples were taken directly prior to release of devils and post-release samples were collected during trapping trips in Narawntapu National Park approximately 2, 4, 8, and 12 weeks following release.

Faecal samples were collected opportunistically and non-invasively during daily cleaning, routine health checks and from traps during routine monitoring by the STDP. Where possible, the identity, sex and age of the animal were noted for each sample. For samples collected during daily cleaning from group pens, only a single sample was collected in order to prevent re-sampling. Following collection, all samples were stored at 4°C.

2.2. DNA extraction and general PCR methods

DNA was extracted from ~150 mg of each faecal sample using the Isolate Fecal DNA kit (Bioline, London, UK) according to the manufacturer's instructions. Extracted DNA was stored at -20°C. All PCR's were performed in conjunction with a negative control (sterile H_2O) and a positive control (DNA extracted from purified *C. parvum* oocysts or *G. lamblia* cysts acquired from Waterborne Inc, USA). For all protocols, secondary PCR products were resolved by electrophoresis on a 1.5% agarose gel containing 2µL of SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, California) and visualized under UV light. Bands were compared to a Hyperladder II DNA marker (Bioline, London, UK) to estimate amplicon size. PCRs were performed in an Eppendorf Mastercycler (Eppendorf, North Ryde, Australia).

2.3. *Cryptosporidium PCR: 18S rDNA, actin and gp60 loci*

For all *Cryptosporidium* protocols, samples were combined with an equal volume of GeneReleaser (BioVentures, Inc., TN, USA) and microwaved for seven minutes in a 500W microwave directly prior to PCR analysis. Samples were initially screened for *Cryptosporidium* at the 18S rDNA locus (~825 bp) using a previously described nested PCR (Vermeulen et al., 2015c). Primary and secondary reaction mixtures contained 2 mM $MgCl_2$, 200 µM dNTPs, 200 nM of each primer, and 2.5 U of Red Hot Taq DNA polymerase (Thermo Scientific, Australia). Reaction conditions comprised an initial denaturation of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 7 min.

In order to confirm positive status and for phylogenetic analysis, samples found positive for *Cryptosporidium* at the 18S rDNA locus were screened at the *actin* locus (~800 bp) using a previously described nested PCR (Sulaiman et al., 2002) with previously introduced modifications (Vermeulen et al., 2015c). Primary and secondary reaction mixtures contained 2 mM $MgCl_2$, 200 µM dNTPs, 2.5 U of Red Hot Taq DNA polymerase (Thermo Scientific, Australia), and 200 nM of each primer. Reaction conditions comprised an initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The conditions for the secondary PCR were identical, except for a higher annealing temperature (54 °C).

Positive samples were also screened at the *gp60* locus (~1000 bp) using a previously described nested PCR (Waldron et al., 2009). Primary and secondary reaction mixtures contained 4 mM $MgCl_2$, 200 nM dNTPs, 200 nM of each primer, and 1 U of Red Hot Taq DNA polymerase (Thermo Scientific, Australia). Reaction conditions comprised an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C 45 s, 58°C 45 s and 72°C for 1 min 30 s, with a final extension at 72°C for 5 min.

2.4. Giardia PCR: β-giardin and gdh loci

DNA from all faecal samples was screened for *Giardia* at the β-giardin (~511 bp) and *gdh* (~432 bp) loci. The β-giardin locus was tested using a previously described nested PCR (Lalle et al., 2005) with a modified secondary forward primer (Delport et al., 2014). Primary and secondary reaction mixtures contained 1.5 mM $MgCl_2$, 200 nM dNTPs, 10 pmol of each primer, and 1 U of Tth DNA polymerase (Promega, USA). Reactions conditions comprised an initial denaturation at 95°C for 15 min followed by 35 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 1 min 30 s, with a final extension at 72 °C for 7 min.

Testing at the *gdh* locus used a previously described semi-nested PCR (Read et al., 2004). Primary and secondary reaction mixtures contained 1.5 mM $MgCl_2$, 200 nM dNTPs, 12.5 pmol of each primer, and 1 U of Tth DNA polymerase (Promega, USA). Reactions conditions comprised an initial cycle of 94°C for 2 min, 56 °C for 1 min and 72 °C for 2 min, 55 cycles of 94 °C for 30 s, 56 °C for 20 s and 72 °C for 45 s, with a final extension at 72 °C for 7 min.

2.5. DNA sequencing, sequence analysis, and phylogenetic analysis

Amplicons for all *Cryptosporidium* and *Giardia* loci were purified using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced in the forward and reverse directions (Ramaciotti Centre for Genomics, Randwick, Australia). Forward and reverse sequences were aligned and assessed manually for quality, and consensus sequences were extracted using Geneious version 8.0.5 (Biomatters, New Zealand). BLAST searches were conducted on consensus sequences for genus confirmation and species identification.

Consensus sequences generated for *Cryptosporidium* and *Giardia* were aligned to reference sequences from GenBank for species identification. For phylogenetic analysis of *Cryptosporidium* at the 18S rDNA locus, aligned sequences were trimmed to equal length and phylogenetic trees were created in Geneious version 8.1.3 using neighbour joining.

The *Cryptosporidium* 18S rDNA reference sequences used are as follows: *C. andersoni* (FJ463171), *C. baileyi* (L19068), *C. bovis* (AY741305), *C. canis* (AF112576), *C. cuniculus* (FJ262765), *C. fayeri* (AF108860), *C. felis* (AF108862), *C. fragile* (EU162751), *C. galli* (HM116388), *C. hominis* (AF093491), *C. macropodum* (AF513227), *C. meleagridis* (AF112574), *C. molnari* (HM243548), *C. muris* (AF093497), *C. parvum* (AF108864), *C. ryanae* (FJ463193), *C. serpentis* (AF151376), *C. suis* (AF108861), *C. ubiquitum* (AF442484), *C. varanii* (AF112573), *C. wrairi* (AF115378), *C. xiaoi* (FJ896053).

The *Giardia gdh* reference sequences used are as follows: *G. duodenalis* A1 (JN982015), *G. duodenalis* AII (L40510), *G. duodenalis* BIII (AF069059), *G. duodenalis* BIV (L40508), *G.*

duodenalis C (U60983), *G. duodenalis* D (U60986), *G. duodenalis* E (DQ182601), *G. duodenalis* F AF069057, *G. duodenalis* H (GU176101.1), *G. ardeae* (AF069060). The *G. peramelis* sequence was obtained via personal communication with Alison Hillman and Amanda Ash at Murdoch University.

2.6. Statistics

Statistical analyses were conducted using R version 3.2.5. Prevalence estimates were calculated by dividing the number of positive samples by the number of devils in the relevant population. For the purpose of calculating prevalence, only the first sampling event was included for devils with repeat samples. The prevalence of *Cryptosporidium* and *Giardia* was compared between population types and between facilities within each population type using a two-tailed Fisher exact test.

3. Results

3.1. Cryptosporidium

3.1.1. Prevalence of Cryptosporidium in the Tasmanian devil

PCR screening identified 29/190 samples as positive for *Cryptosporidium* at the 18S rDNA locus (Table 1). These 29 samples came from 27 individual animals; two juvenile devils from Bridport Free-range Enclosure tested positive twice, with 21 days in-between sampling events. There was no significant difference in prevalence (p = 1.000) between the intensive captive (8.6%; 95% C.I. = 3.2 – 19.7%) and free-range captive population types (12.5%; 95% C.I. = 5.9 – 23.7%). However, the prevalence in the wild population (37.9%; 95% C.I. = 21.3 – 57.6%) was significantly higher than the prevalence in the captive population types (p = 0.002).

At the population level, there was no significant difference in prevalence (p=0.725) between the Table Mountain and Narawntapu wild populations and the Bridport free-range captive population (33.3 – 50.0%). There was no significant difference (p = 0.676) in *Cryptosporidium* prevalence between the Freycinet, Devil Ark, Healesville, Cressy, Monarto, Taronga, and Western Plains Zoos populations (0.0 – 15.0%). However, the 37.5% prevalence of *Cryptosporidium* in the Bridport free-range population was significantly higher than the prevalence in the other captive populations (p = 0.002).

To confirm positive status and to allow for *Cryptosporidium* species identification, samples that were positive at the 18S rDNA locus were also screened at the *actin* and *gp60* loci. At the *actin* locus, 2/29 positive samples were confirmed positive for *Cryptosporidium*. None of the 29 samples that were positive at the 18S rDNA locus amplified at the *gp60* locus, despite a strong amplicon being produced for the *C. parvum* positive control.

3.1.2. Cryptosporidium species identification and phylogenetic analysis:

Of the samples that were positive for *Cryptosporidium* at the 18S rDNA locus, 25/29 were 100% identical to each other and BLAST searches revealed that these samples represent a novel genotype of *Cryptosporidium* (*Cryptosporidium sp TD23, TD26, TD27, TD28, TD29, TD30, TD31, TD32, TD63, TD68, TD86, TD98, TD102, TD103, TD104, TD119, TD121, TD132, TD147, TD148, TD172, TD177, TD185, TD187, TD188,* Figure 1). The closest match for this genotype (96.5%) was a *Cryptosporidium* environmental isolate from storm water in New York (GenBank: AY737575.1). One further sample, for which only a partial reverse sequence was obtained, had 100% alignment with this novel genotype over a span of 429 nucleotides. Samples containing the novel genotype were from wild, intensive captive, and free-range captive population types, as well as from two vaccine-trial devils, 39 and 47 days post-release, respectively; no other samples were collected from the first of these devils, but for the second devil, a sample obtained 14 days post-release was negative for *Cryptosporidium*, as was a sample obtained 77 days post-release. This novel genotype was amplified on both occasions for the two juvenile devils that were positive for *Cryptosporidium* on re-sampling, with 21 days in between sampling events.

BLAST searches revealed that one of the 29 18S rDNA-positive samples was a 99.4% match to *C. fayeri* (GenBank: KP730318.1) (*Cryptosporidium sp TD20*, Figure 1). The sequence for this sample was also highly similar to *C. fayeri* at the *actin* locus (GenBank: KP730322.1), with 99.6% match nucleotide identity. The *C. fayeri* positive sample originated from a wild devil from Table Mountain. One other sample amplified at the *actin* locus where it was also highly similar to *C. fayeri,* with 99.5% nucleotide identity. This sample originated from a vaccine-trial devil, 47 days post-release, which was found to harbour the novel genotype of *Cryptosporidium* at the 18S rDNA locus.

A single *Cryptosporidium* 18S rDNA-positive sample from a wild devil from Table Mountain was a 99.3% match for *C. muris* isolates from both mice and bactrian camels (GenBank: KP994665.1, EU245044) (*Cryptosporidium sp TD19,* Figure 1).

The last positive sample at the 18S rDNA locus was a 99.6% match for *C. galli* (GenBank: KU744848.1). This was a pre-release sample from a released devil; no post-release samples were obtained for this devil (*Cryptosporidium sp TD43,* Figure 1).

3.2 Giardia

3.2.1. Prevalence of Giardia in Tasmanian devils

PCR screening identified 8/190 samples as positive for *Giardia* at either the *β-giardin* or *gdh* loci (Table 1). Of these eight samples, one amplified at both the *β-giardin* or *gdh* loci; the

remaining seven positive samples amplified only at the *gdh* locus. The eight positive samples came from eight different devils. There was no significant difference ($p = 1.000$) in the prevalence of *Giardia* between the intensive captive (0.0%; 95% C.I. = 0.0 – 7.7%) and free-range captive population types (1.6%; 95% C.I. = 0.0 – 9.5%). However, the prevalence in the wild population type (24.1%; 95% C.I. = 11.0 – 43.9%) was significantly higher than the captive population types ($p < 0.001$).

At the population level, there was no significant difference in *Giardia* prevalence ($p = 1.000$) between the Table Mountain and Narawntapu wild populations (8.3 – 25.0%). Likewise, there was no significant difference in prevalence ($p = 0.262$) between any of the captive populations (0.0 – 8.3%).

3.2.2. *Giardia species identification and phylogenetic analysis:*

PCR screening at the *β-giardin* and *gdh* loci identified eight samples as positive for *Giardia* (Table 1). Only one sample was positive at the *β-giardin* locus; a BLAST search found this sample to be a 91.4% match for *G. duodenalis* (GenBank: KP687756.1).

All eight positive samples amplified at the *gdh* locus. Four of these samples were 100% identical to each other, (*Giardia sp TD23, TD38, TD30, TD64*, Figure 2) and this group also included the sample that was positive at the *β-giardin* locus. BLAST searches indicated that these four samples are likely represent a novel genotype of *Giardia*; their closest match (89.4%) was *G. duodenalis*, assemblage B isolated from mussels off the coast of central California (GenBank: KF294079.1).

Two of the other positive samples were also identical to each other, with 100% nucleotide agreement, and a third positive sample had 99.8% nucleotide agreement with these two samples (*Giardia sp TD23, TD53, TD56*, Figure 2). BLAST searching indicated that these three samples are likely to represent another novel genotype, with the closest match (88.1%) being *G. duodenalis*, assemblage A isolated from humans in South India (GenBank: JN616252.1). This novel genotype and the novel genotype above had 94.3% nucleotide agreement over a stretch of 402 nucleotides.

The final positive sample was found to be a 100.0% match to *G. duodenalis*, assemblage B (GenBank: JX448643.1) (*Giardia sp TD80*, Figure 2). Alignment with GenBank reference sequences for *G. duodenalis* assemblage BIV revealed that this amplicon was only one nucleotide different from *G. duodenalis* BIV (GenBank: L40508). This sample originated from a devil in the Freycinet free-range captive population.

Table 1

Results and species identification for *Cryptosporidium* and *Giardia* in different populations of Tasmanian devils. For the vaccine-trial devils, the 13 pre-release samples each represent a different individual, while the eight post-release samples came from four individuals, three of which did not provide a pre-release sample.

Population Type	Population		No. of faecal samples	No. of animals	*Cryptosporidium*		*Giardia*	
					18s rDNA	*actin*	B-giardin	*gdh*
Intensive Captive	Monarto Zoo		7	7	0	0	0	0
	Healesville Sanctuary		20	20	3	0	0	0
	Taronga Zoo		3	3	0	0	0	0
	Western Plains Zoo		10	10	0	0	0	0
	Cressy breeding facility		18	18	2	0	0	0
		Total	58	58	5	0	0	0
Free-range Captive	Devil Ark		36	36	2	0	0	0
	Freycinet Peninsula		14	12	0	0	0	1
	Bridport		22	16	6(8)*	0	0	0
		Total	72	64	10	0	0	1
Wild	Narawntapu National Park		31	21	7	0	1	5
	Table Mountain		8	8	4	1	0	2
		Total	39	29	11	1	1	7
Vaccine trial devils	Pre-release		13	13	1	0	0	0
	Post-release		8	4 (3 new)	2	1	0	0
		Total	21	17	3	1	0	0
		Total	190	167	29	2	1	8

*Indicates repeat sampling; for the Bridport population, eight samples from six individual devils were positive for *Cryptosporidium*.

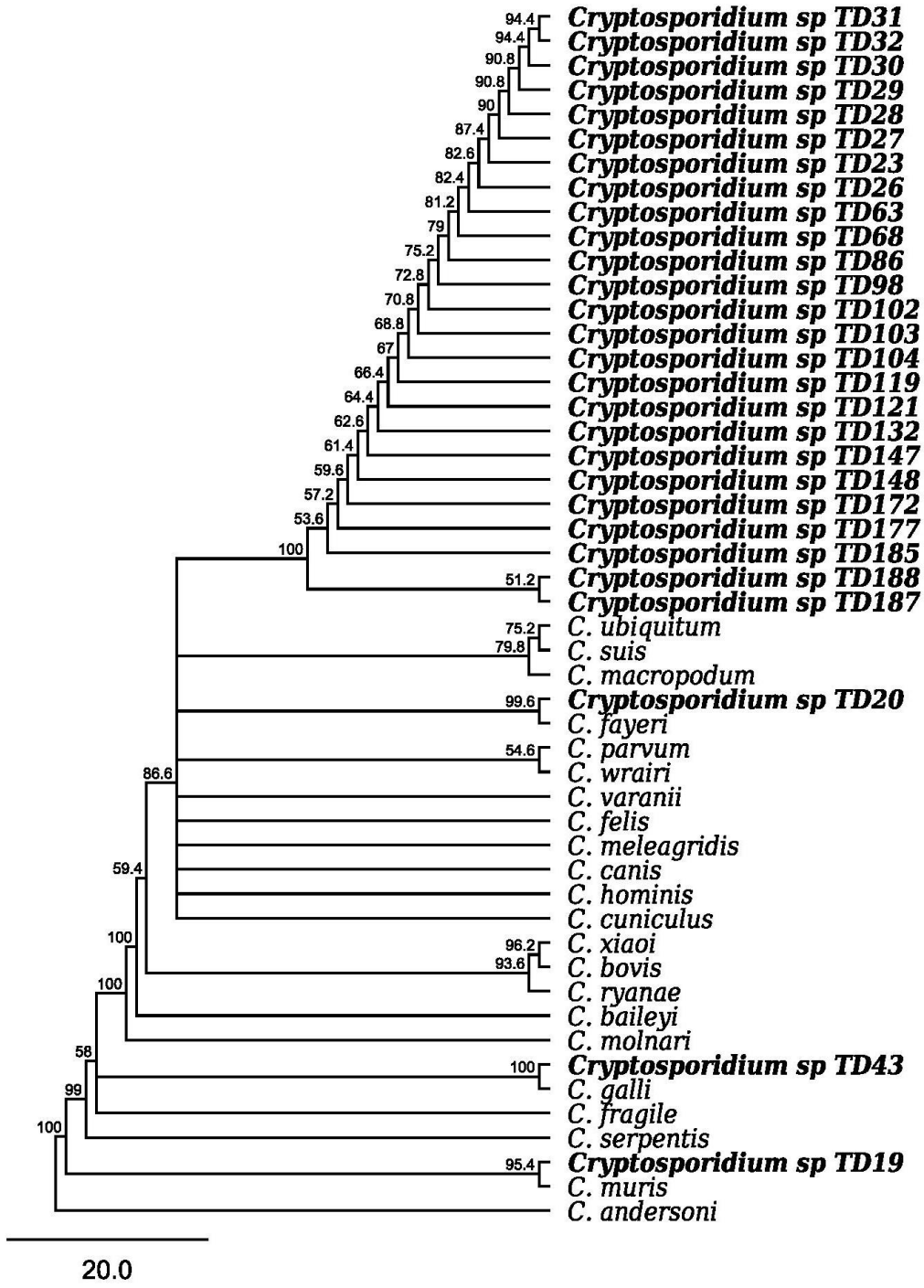

Figure 1

Inferred phylogeny of *Cryptosporidium* 18S rDNA consensus sequences (709 nucleotides). The *Cryptosporidium* 18S rDNA reference sequences used for this phylogeny are as follows: *C. andersoni* (FJ463171), *C. baileyi* (L19068), *C. bovis* (AY741305), *C. canis* (AF112576), *C. cuniculus* (FJ262765), *C. fayeri* (AF108860), *C. felis* (AF108862), *C. fragile* (EU162751), *C. galli* (HM116388), *C. hominis* (AF093491), *C. macropodum* (AF513227), *C. meleagridis* (AF112574), *C. molnari* (HM243548), *C. muris* (AF093497), *C. parvum* (AF108864), *C. ryanae* (FJ463193), *C. serpentis* (AF151376), *C. suis* (AF108861), *C. ubiquitum* (AF442484), *C. varanii* (AF112573), *C. wrairi* (AF115378), *C. xiaoi* (FJ896053).

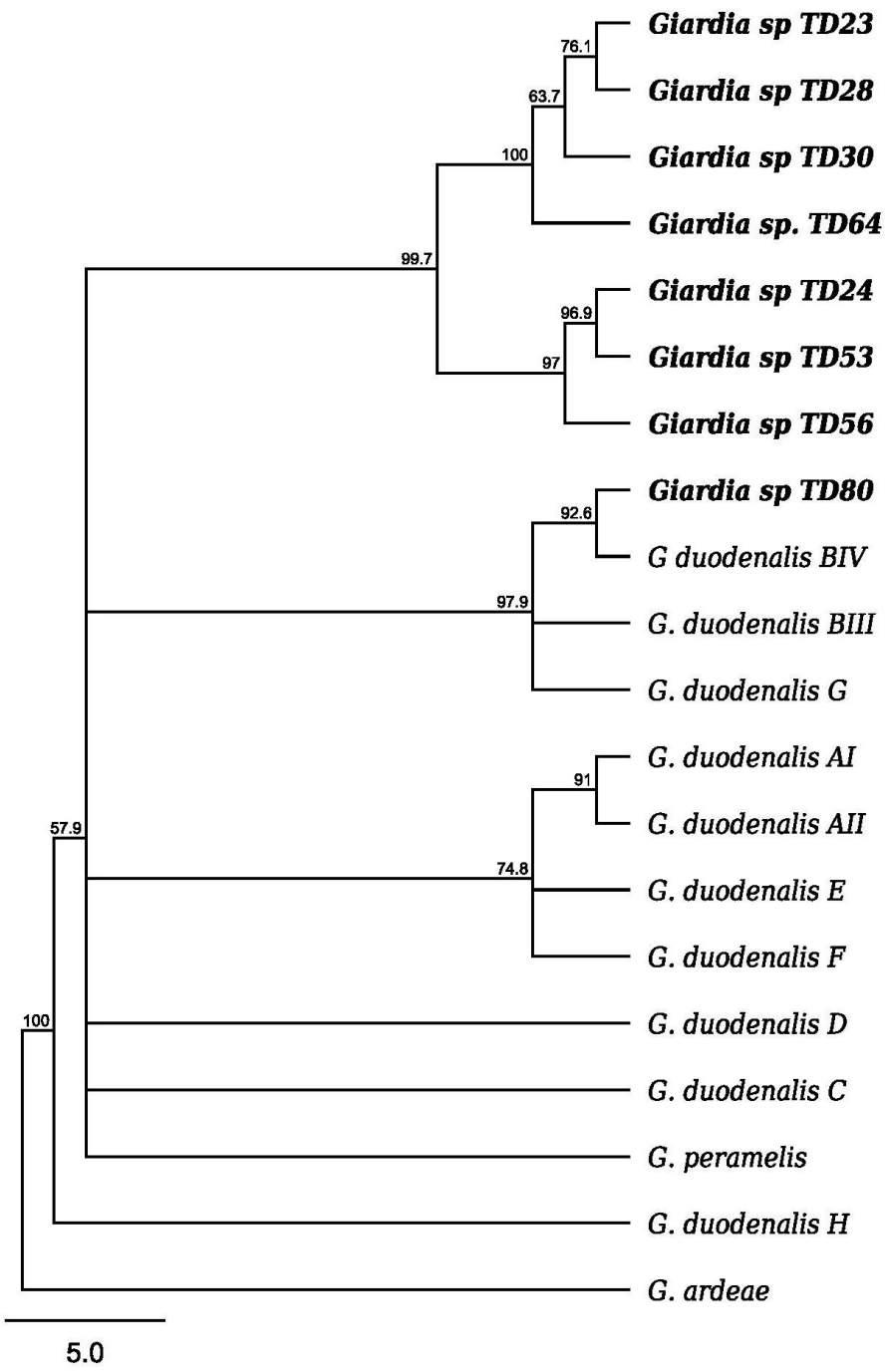

Figure 2

Inferred phylogeny of *Giardia gdh* sequences (199 nucleotides). The *Giardia gdh* reference sequences used for this phylogeny are as follows: *G. duodenalis* A1 (JN982015), *G. duodenalis* AII (L40510), *G. duodenalis* BIII (AF069059), *G. duodenalis* BIV (L40508), *G. duodenalis* C (U60983), *G. duodenalis* D (U60986), *G. duodenalis* E (DQ182601), *G. duodenalis* F AF069057, *G. duodenalis* H (GU176101.1), *G. ardeae* (AF069060). The *G. peramelis* sequence was obtained via personal communication with Alison Hillman and Amanda Ash at Murdoch University.

4. Discussion

This study provides the first report of *Cryptosporidium*, and the first published molecular characterisation of *Giardia,* in Tasmanian devils. *Cryptosporidium* was detected in wild, intensive captive and free-range captive devils, while *Giardia* was only detected in wild and free-range captive devils. Both *Cryptosporidium* and *Giardia* were significantly more prevalent in wild compared with captive populations of devils. One novel genotype of *Cryptosporidium* was identified, as well as samples with high sequence similarity to *C. fayeri*, *C. muris*, and *C. galli*. Two novel genotypes of *Giardia* were identified, and *G. duodenalis* assemblage BIV was identified in a single free-range captive devil.

Both *Cryptosporidium* and *Giardia* were significantly more prevalent in wild compared with captive populations of Tasmanian devils. This could be due to several factors. *Cryptosporidium* and *Giardia* have direct life cycles that rely on contact with infected faecal material or contaminated food or water for transmission. Captive devils, particularly in intensive facilities, are provided with water from the same water mains that supply humans around Australia and hence would have low risk of contracting *Cryptosporidium* and *Giardia* from this source. Additionally, captive devils in intensively managed facilities are sometimes housed individually which would further preclude the transmission of *Cryptosporidium* and *Giardia.* Finally, enclosures are routinely cleaned of faecal material in both intensive and free-range captive facilities which would reduce the amount of infectious material available for parasite transmission and so could also be a factor in the lower prevalence rates seen in captive compared with wild devils. These findings could have health implications for captive devils that are subsequently released into the wild, as they may have little or no acquired immunity against parasites which they are likely to be exposed to in the wild. Further study of released devils and the changes that occur in their parasite communities are warranted to investigate this issue.

4.1. Cryptosporidium

Cryptosporidium has previously been described in a range of Australian marsupials. This study found a 37.9% prevalence of *Cryptosporidium* in wild Tasmanian devils, which is higher than prevalence estimates in other Australian marsupials which range from 6.7% to 12.2% (Hill et al., 2008; McCarthy et al., 2008; Power et al., 2005; Vermeulen et al., 2015a; Yang et al., 2011). Only two samples amplified successfully at the *actin* locus, and no samples amplified at the *gp60* locus. This failure to amplify at confirmatory loci may be due to both *actin* and *gp60* being single copy loci compared with the multi-copy 18S rDNA locus, and hence having less template DNA available to amplify (Hill et al., 2008; Power et al., 2009). Inefficient primer binding for the novel *Cryptosporidium* genotype may also account for inability to amplify at actin and *gp60* loci.

Additionally, the *gp60* locus is highly polymorphic and only a few *Cryptosporidium* species have been characterized at this locus (Burton et al., 2010; Feng et al., 2011; Li et al., 2014; Power et al., 2009). Future studies of *Cryptosporidium* in Tasmanian devils should attempt to amplify different loci, for example HSP70 (Sulaiman et al., 2000). Future studies could also attempt to increase the amount of template DNA available for amplification by isolating and concentrating oocysts from faecal samples that are positive at the 18S rDNA locus, and extracting DNA from these concentrated oocysts prior to confirmatory PCR analysis.

The majority of *Cryptosporidium*-positive samples appear to represent a novel genotype, though it will be important to confirm this via amplification and sequencing at other loci, and by performing oocyst counts and morphological analysis of oocysts. The finding of a novel genotype of *Cryptosporidium* in Tasmanian devils is not entirely unexpected; it is not unusual for novel genotypes of *Cryptosporidium* to be identified when wildlife hosts are investigated for *Cryptosporidium* for the first time (Ryan and Power, 2012; Ziegler et al., 2007).

One Tasmanian devil sample was highly similar to *C. fayeri* at the 18S rDNA locus, and two were highly similar at the *actin* locus *C. fayeri* is a marsupial species of *Cryptosporidium* and has previously been identified in a range of marsupial hosts from mainland Australia, including macropods, koalas, and bandicoots (Power, 2010). *C. fayeri* is thought to be host-adapted, as infections in marsupial hosts have not been associated with disease (Power, 2010). However, *C. fayeri* was identified as the cause of a human clinical case of cryptosporidiosis in 2009 (Waldron et al., 2010), and so the finding of this species in Tasmanian devils could have health implications for people working closely with devils. The fact that one sample produced an amplicon with high sequence similarity to *C. fayeri* at the *actin* locus, but appeared to harbor the novel genotype at the 18S rDNA locus could be indicative of a mixed infection.

C. muris, which was identified in a single Tasmanian devil sample, is a species of *Cryptosporidium* with broad host-specificity. Rodents (*Mus musculus* and *Rattus spp.*) and bactrian camels (*Camelus bactrianus*) serve as the primary hosts for *C. muris* (Xiao et al., 2004), and this species also infects immunocompromised humans, a range of other eutherian mammals, and ostriches (*Struthio camelus*) (Mynářová et al., 2016; Santín et al., 2005; Tiangtip and Jongwutiwes, 2002; Wagnerová et al., 2015; Xiao et al., 1999) (Qi et al., 2014). Notably, *C. muris* has been documented in one other Australian marsupial, the bilby (*Macrotis lagotis*) (Warren et al., 2003); multiple infections occurred in a captive colony of bilbies and were traced to house mice entering the colony (Warren et al., 2003). *C. muris* is not a genetically uniform species, and studies have identified multiple genetically distinct subtypes (Hikosaka and Nakai, 2005; Wang et al., 2012; Xiao et al., 1999). The 0.7% variation found between the isolate identified here and recorded

isolates of *C. muris* is comparable to the amount of variation seen between other recorded isolates of *C. muris* at the 18S rDNA locus; for example isolates from a house mouse (*Mus musculus*) and a large Japanese field mice (*Apodemus speciosus*) were only 99.5% similar (GenBank: AF093498 and AY642591).

C. galli, a *Cryptosporidium* species that causes clinical disease in chickens and finches, among other birds (Ryan et al., 2003) was identified in one Tasmanian devil sample. Unfortunately this sample did not amplify at the *actin* or *gp60* loci, and future work should attempt amplification at further loci to determine whether it represents a novel genotype or *C. galli*. If this isolate is confirmed as *C. galli*, it will be the first time this species has been identified in a mammalian host. However, it is also possible that *C. galli* DNA may have been amplified from a devil passively passaging oocysts that it had ingested and not from infection. Captive devils are occasionally fed chicken (pers. comm. Olivia Barnard), and are also exposed to wild birds in their enclosures, two possible transmission or contamination routes for *C. galli*. Though this would be the first example of *C. galli* in a mammalian host, *C. meleagradis*, a species of *Cryptosporidium* that infects birds and humans (Appelbee et al., 2005), has recently been identified in the brush-tailed rock wallaby (*Petrogale pencillata*), another Australian marsupial (Vermeulen et al., 2015a).

4.2 Giardia

Giardia has previously been described in a range of Australian marsupials. The estimates of prevalence of *Giardia* in wild Tasmanian (24.1%) is higher than prevalence estimates for mainland Australian marsupials (range 1.3 - 13.8%)(Adams et al., 2004; Ng et al., 2011; Thompson et al., 2010; Vermeulen et al., 2015b), but sits within the range of prevalence estimates for Tasmanian marsupials (6.25 - 61.5%) (Bettiol et al., 1997). The 24.1% prevalence estimate for wild devils was also higher than previous prevalence estimates of *Giardia* in devils of 8.3% (n = 12) (Davies, 1995) and 6.0% (n = 32) (Kettlewell et al., 1998). This higher estimate is likely due to the use of molecular techniques in this study, which are far more sensitive than the faecal flotation methods used in prior studies of *Giardia* in Tasmanian devils (Morgan et al., 1998; McGlade et. al., 2003).

Molecular studies of *Giardia* in other Australian marsupials have found that the quenda (*Isoodon obesulus*) hosts a novel species, *G. peramelis*, while other Australian marsupials have been shown to host various assemblages of *G. duodenalis* (syn. *G. lamblia* and *G. intestinalis*). *G. duodenalis* is the species responsible for most human giardiasis, and rather than representing a single species, molecular studies of *G. duodenalis* have revealed it to be a species-complex that is divided up into eight genetic assemblages (A-H), which are further divided into sub-assemblages (ie. AI, AII) with varying pathogenicity and host-specificity (Feng and Xiao, 2011). This study identified two novel genotypes of *Giardia* in Tasmanian devils, and also identified *G. duodenalis* BIV in a single captive

devil. Further work is required to characterise the two novel genotypes. Analysis at other loci, such as the ITS1-5.8s-ITS2 would enable comparison to *G. peramelis*, the *Giardia* species found in the Quenda (Hillman et al., 2016). Tasmanian devil faecal samples should also be subjected to microscopic analysis in order to determine cyst burdens and allow morphological characterisation of cysts.

G. duodenalis BIV, a zoonotic assemblage of *G. duodenalis* with broad host-specificity, was identified in a sample from a free-range captive devil. Assemblage BIV has previously been described in a number of Australian marsupial species, including another species of dasyurid, the spotted-tailed quoll (*D. maculatus*) (Thompson et al., 2008; Vermeulen et al., 2015b). The identification of this assemblage in Tasmanian devils could imply spill-over from humans to Tasmanian devils, either directly or indirectly. Such human-devil transmission could result from direct contact, but would more likely be the result of human environmentalcontamination.

5. Conclusions

This study reports the presence of novel genotypes of *Cryptosporidium* and *Giardia* in Tasmanian devils. A comparison of parasite prevalence between captive and wild devils showed a significantly higher prevalence of both *Cryptosporidium* and *Giardia* in wild devils. Isolates with high similarity to *C. fayeri, C. muris,* and *C. galli* were also identified. *G. duodenalis* BIV, a zoonotic genotype of *Giardia,* was identified in a captive devil, indicating that human-devil transmission may be occurring. This finding means that further investigation of *Giardia* in captive Tasmanian devils is definitely warranted, as is investigation of other possible human specific pathogens. Overall, the findings suggest that conservation management may be changing host-parasite interactions in the Tasmanian devil, as evidenced by the lower prevalence of both *Cryptosporidium* and *Giardia* in captive compared with wild devils. These findings should help to guide the Save the Tasmanian Devil Program in their goal to conserve devil-associated symbionts and parasites.

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CHAPTER 3

PARASITE DIVERSITY IN THE TASMANIAN DEVIL – A COMPARATIVE STUDY OF WILD AND CAPTIVE DEVILS

Abstract

Conservation management may impact host-parasite interactions. This study investigated the impact of conservation management on parasite prevalence and diversity in an endangered Australian marsupial, the Tasmanian devil. Tasmanian devils are threatened by devil facial tumour disease, a lethal transmissible cancer. As a result of this threat, Tasmanian devils are the focus of the Save the Tasmanian Devil Program (STDP), a conservation program which integrates the captive breeding of devils and monitoring of wild devil populations. Tasmanian devils are known to host a variety of parasites, including nematodes, platyhelminthes, protozoa, and ectoparasites, however, we have limited knowledge of the majority of these parasites in this host. This study aimed to provide baseline data on gastrointestinal parasites in the Tasmanian devil, and to compare parasite prevalence and diversity between wild and captive devils. Six types of parasite were identified: *Baylisascaris tasmaniensis*; a taeniid species, presumed to be *Anoplotaenia dasyuri*; coccidia; a strongylid species, thought to be *Woolleya sarcophili*; *Cryptosporidium*; and *Giardia*. There was no significant difference in the prevalence of the taeniid species between wild and captive devils, but *B. tasmaniensis*, *Cryptosporidium* and *Giardia* were all significantly more prevalent in wild compared with captive devils, while coccidia and the strongylid species were more prevalent in captive compared with wild devils ($p < 0.05$). Wild devils were significantly more likely to harbour at least one parasite species compared with captive devils ($p < 0.05$). These findings suggest that conservation management may be changing host-parasite interactions in the Tasmanian devil, and should help to guide the STDP in their goal to maintain devil-associated symbionts and parasites.

Keywords: Tasmanian devil, parasite diversity, conservation management

1. Introduction

The Tasmanian devil (*Sarcophilus harrisii;* Dasyuridae: Marsupalia) is the largest extant carnivorous marsupial. Though once prevalent across mainland Australia, Tasmanian devils have been restricted to the island of Tasmania for the last 3000 - 4000 years (Brown, 2006). Devils can be found state-wide in Tasmania, though their habitat is becoming increasingly fragmented by urban and agricultural development (Jones et al., 2004). Tasmanian devils are specialised scavengers with an indiscriminate diet (Pemberton et al., 2008), and play an important role as the apex predator in Tasmania (Hollings et al., 2014). They share much of their habitat with two smaller dasyurid species, the eastern quoll (*Dasyurus viverrinus*) and spotted-tailed quoll (*D. maculatus*) (Jones and Barmuta, 1998, 2000).

Tasmanian devils are currently threatened by devil facial tumour disease (DFTD), an invariably lethal transmissible cancer (Bender et al., 2014). DFTD first emerged in 1996, and has since spread through more than 85% of the Tasmanian devil population (Bender et al., 2014). In the face of this threat, devils are the subject of a conservation program known as the Save the Tasmanian Devil Program (STDP) (DPIPWE, 2014b). This program integrates management of wild populations and captive breeding of devils in a range of facilities in Tasmania and mainland Australian (DPIPWE, 2014b). Recently, the program has also begun establishing DFTD-free wild populations on islands and peninsulas off of Tasmania that are isolated from the DFTD-front (DPIPWE, 2014a, 2015a). A program objective of the STDP is to conserve commensal, symbiotic and parasitic organisms associated with Tasmanian devils (DPIW and ARAZPA, 2007), however there has not yet been any systematic evaluation of how the program may be changing devil host-parasite interactions.

Tasmanian devils are known to host a suite of parasitic species, including nematodes, platyhelminthes, protozoa, and ectoparasites (Beveridge and Spratt, 2003). However, little is known about the current status of many of these parasites in the Tasmanian devil. The massive DFTD-induced population decline, combined with increasingly fragmented devil populations, may have already resulted in the extirpation of some of these parasitic species. Additionally, the STDP could itself be causing changes to Tasmanian devil parasite diversity via anthelmintic treatment of captive devils and the isolation of captive devils from parasite infective stages.

Fifteen gastrointestinal parasites representing broad taxonomic classes have been described in the Tasmanian devil. Tasmanian devils are known to host four gastrointestinal nematode species: *Baylisascaris tasmaniensis*, *Woolleya sarcophili*, *Physaloptera sarcophili*, and *Cyathospirura seurati*. *B. tasmaniensis* is an ascarid nematode for which Tasmanian devils, eastern quolls (*D. viverrinus*), and spotted-tailed quolls (*D. maculatus*) are the definitive host (Obendorf, 1993). *W. sarcophili* is the only known strongylid from Tasmanian devils; this species has been reported to

have a low prevalence in devils and has not been fully described morphologically (Humphery-Smith and Durette-Desset, 1981). Tasmanian devils serve as the definitive host for *Physaloptera sarcophili* along with northern quolls (*D. hallucatus*) (Johnston and Mawson, 1940). The last gastrointestinal nematode, *C. seuratis*, is found in the stomachs of Tasmanian devils and other dasyurids as well as eutherian carnivores including cats and foxes (Beveridge and Spratt, 2003). Tasmanian devils have been recorded as hosting six cestode species: *Spirometra erinacei*, *Anoplotaenia dasyuri*, *Dasyurotaenia robusta*, *Taenia pisiformis*, *Taenia ovis*, and *Taenia hydatigena* (Beveridge, 1984; Gregory, 1972, 1976; Gregory et al., 1975; Spratt et al., 1991). The first cestode in this list, *S. erinacei*, is a cosmopolitan species with dog, cat and fox definitive hosts; Tasmanian devils serve as an accidental intermediate host for this cestode (Beveridge and Spratt, 2003). The remaining four cestodes known from Tasmanian devils are taeniid species. While Tasmanian devils are one of main definitive hosts for *A. dasyuri* (Gregory et al., 1975), and the only known definitive host for *D. robusta* (Beveridge, 1984), the last three species in this list have eutherian carnivores as their main host (Gregory, 1972, 1976; Spratt et al., 1991). Two gastrointestinal protozoa have been described from Tasmanian devils: *Giardia spp*. and *Sarcocystis spp*. *Giardia* is a ubiquitous genus of flagellated protozoan parasite, comprising species that infect a broad range of vertebrate hosts (Appelbee et al., 2005), while *Sarcocystis* is a genus of cyst-forming protozoan parasite (Tenter, 1995). Neither of these protozoa have been identified or classified beyond the genus level in Tasmanian devils (Davies, 1995; Kettlewell et al., 1998; Munday et al., 1978).

Parasites are increasingly being recognised as important regulators of ecological communities (Hudson et al., 2006), and parasite conservation is an important factor in biodiversity maintenance (Dougherty et al., 2015). Moreover, the STDP has explicitly indicated the conservation of commensal, symbiotic and parasitic organisms associated with Tasmanian devils as a program goal (DPIW and ARAZPA, 2007). This study aimed to provide baseline data on the prevalence and diversity of Tasmanian devil gastrointestinal parasites and to examine the possibility that changes may be occurring within devil parasite-communities as a result of the STDP.

2. Materials and methods

2.1. Sample collection

A total of 190 faecal samples were collected from 167 Tasmanian devils (Table 1). These samples came from devils in three different "population types": intensively managed captive, free-range captive, and wild. Each of these population types were made up of multiple populations such that there were five intensively managed captive populations (Monarto Zoo, Healesville Sanctuary, Taronga Zoo, Western Plains Zoo, and the Cressy STDP breeding facility), three free-range captive populations (Devil Ark and the STDP free-range enclosures at Bridport and on the Freycinet

Peninsula), and two wild populations (Table Mountain and Narawntapu National Park). Note: we use the word "population" to denote a group of animals living in the same location/facility. All samples were collected between July 2015 and February 2016, with the exception of 18 samples from Devil Ark that were collected in June 2014. Wild samples were collected from July 2015 – February 2016, free-range captive samples were collected from November 2015 – February 2016, and intensive captive samples were collected from October 2015 – February 2016. Additionally, pre- and post-release faecal samples were collected from 16 devils that were vaccinated with a trial vaccine against DFTD and released into Narawntapu National Park in September 2015 (DPIPWE, 2015b). The vaccine-trial devils were bred in captivity as part of the insurance population and were housed in the Freycinet and TasZoo free-range captive enclosures for 4 – 6 months prior to release (pers. comm. Samantha Fox). Pre-release samples were taken directly prior to release of devils and post-release samples were collected during trapping trips in Narawntapu National Park approximately 2, 4, 8, and 12 weeks following release.

Faecal samples were collected opportunistically and non-invasively during daily cleaning, routine health checks and from traps during routine monitoring by the STDP. Where possible, the identity, sex, age and DFTD-infection status of the devil were noted for each sample. Following collection, all samples were stored at 4°C.

2.2. Faecal flotation and microscopy

Samples were processed using zinc sulfate centrifugal faecal flotation (Zajac and Conboy, 2012). Slides were scanned for the presence of helminth eggs and protozoal oocysts at 100X magnification, and findings were confirmed at 400X magnification. Microscopy was performed using an Olympus CHA light microscope. Parasite stages were digitally photographed and measured at 400X magnification under a BX53 microscope equipped with DIC optics and CellSens interface software (Olympus, Shinjuku, Japan). Parasite eggs and oocysts were identified based on morphological descriptions and photographs, when available. Average dimensions for helminth eggs were calculated from measurements of at least ten eggs for each helminth type.

2.3. Molecular detection of Giardia and Cryptosporidium spp.

Prevalence data for *Giardia* and *Cryptosporidium spp*. was used from a molecular study conducted on the same sample set. For the methods used, see Chapter 2: Molecular characterization of *Giardia* and *Cryptosporidium* from Tasmanian devils.

2.4. Statistics

Statistical analyses were performed using R Version 3.2.5. Prevalence estimates for each parasite type were compared between population types and individual populations using a Fisher exact test. For the purpose of calculating prevalence, only the first sampling event was included for devils with

repeat samples. In order to compare parasite diversity, the mean number of parasites per devil was compared between population types, and between facilities within population types, using the non-parametric Kruskal-Wallis rank sum test.

Table 1

Distribution of Tasmanian devil faecal samples analysed from different populations. For the vaccine trial devils, the 13 pre-release samples each represent a different individual devil, while the 8 post-release samples came from 4 different devils, three of which did not provide a pre-release sample.

Population Type	Population Name/Collection Site/Time		No. of faecal samples	No. of animals
Intensive Captive	Monarto Zoo		7	7
	Healesville Sanctuary		20	20
	Taronga Zoo		3	3
	Western Plains Zoo		10	10
	Cressy breeding facility		18	18
		Total	58	58
Free-range Captive	Devil Ark		36	36
	Freycinet Peninsula		14	12
	Bridport		22	16
		Total	72	64
Wild	Narawntapu National Park		31	21
	Table Mountain		8	8
		Total	39	29
Vaccine trial devils	Pre-release		13	13
	Post-release		8	4 (3 new)
		Total	21	17
		Total	190	167

3. Results

3.1. Parasite prevalence data

Faecal flotation and microscopy identified four "types" of parasite: *B. tasmaniensis*; a strongylid species (presumed to be *W. sarcophili)*; a taeniid species (presumed to be *A. dasyuri)*; and several unidentified species of coccidia, some of which were *Isospora spp*. (Figure 1.) Mite eggs were also identified in a number of samples, but it was not possible to determine whether they were the eggs of devil mites or the mites of a prey species and so these results have not been included in the analysis. Targeted PCR analysis detected two further parasites, *Cryptosporidium* and *Giardia*. Of the six types of parasite detected, *Giardia* was only detected in wild and free-range captive devils, the strongylid species was detected only in captive intensive devils, while the remaining three parasite types were detected in all population types. (Table 2).

B. tasmaniensis eggs (Figure 1, 65 x 70 µm) were identified in 39/190 samples. There was no significant difference in prevalence ($p = 1.000$) between the intensive captive (12.1%) and free-range captive population types (10.9%). However, the prevalence of *B. tasmaniensis* was significantly higher ($p < 0.001$) in the wild population type (34.5%). At the population level, there was no significant difference in the prevalence of *B. tasmaniensis* ($p =0.526$) between the Monarto, Taronga, Devil Ark, Western Plains Zoo, and Healesville captive populations (0.0 – 5.0%). However, these populations had a significantly lower prevalence than the Cressy, Freycinet, and Bridport captive populations (18.8 – 33.3%). There was no significant difference in prevalence ($p = 0.816$) between the Table Mountain, and Narawntapu wild populations, and the Cressy, Freycinet, and Bridport captive populations (18.8 – 37.5%) (Table 2).

Eggs of a taeniid species (Figure 1, 25 x 30 µm), presumed to be *A. dasyuri*, were identified in 12/190 samples. There was no significant difference in prevalence ($p =0.170$) for this parasite between wild (10.3%) intensive captive (1.7%), and free-range captive population types (4.7%). Furthermore, there was no significant difference in prevalence between any of the individual populations ($p = 0.26$), with prevalence estimates ranging from 0.0% to 12.5% (Table 2).

Coccidial oocysts were identified in 33/190 samples (Figure 1). At least three different species of coccidia were detected, but for the purpose of analysis their data has been combined. Additionally, though *Cryptosporidium* is classified as a coccidian species, data for this parasite has been analysed separately (see below) due to the different detection method used. There was no significant difference ($p = 0.07$) in the prevalence of coccidia between the intensive captive (20.7%) and free-range captive population types (25.0%). However, the prevalence of coccidia was significantly lower in the wild population type (6.9%) compared with the captive population types ($p = 0.02$). At the population level there was no significant difference ($p = 0.289$) in the prevalence of coccidia

between Narawntapu, Bridport, Freycinet, Healesville, Western Plains, and Monarto populations (0.0 – 16.7%). However, the prevalence of coccidia at Table Mountain, Devil Ark, Taronga and Cressy was significantly higher ($p < 0.001$) than the other seven populations (25.0 – 38.8%). There was no significant difference in prevalence between the Table Mountain, Cressy, Taronga, and Devil Ark ($p = 0.938$) (Table 2).

Eggs of a strongylid species (Figure 1, 70 x 35 µm), thought to be *W. sarcophili*, were identified in 4/190 samples. The four positive samples all came from animals housed in intensive captive populations; three samples were from the Cressy breeding facility, and one sample was from Healesville. The prevalence of this strongylid species was significantly higher ($p = 0.0483$) in the intensive captive (6.9%) compared with the free-range captive population type (0.0%). However, there was no significant difference ($p = 0.297$) between the prevalence in the intensive captive and wild population types (0.0%) or between the wild and free-range captive population types ($p = 1.000$). At the population level, the prevalence of this strongylid species did not significantly differ ($p = 0.328$) between the Cressy and Healesville intensive captive populations (5.0 – 16.7%). However, the prevalence was significantly higher in these two populations compared with the 0.0% prevalence seen in all of the other populations ($p = 0.0035$) (Table 2).

PCR screening identified 29/190 samples as positive for *Cryptosporidium.* There was no significant difference in prevalence ($p = 1.000$) between the intensive captive (8.6%) and free-range captive population types (12.5%). However, the prevalence in the wild population (37.9%) was significantly higher than the prevalence in the captive population types ($p = 0.002$). At the population level, there was no significant difference in prevalence (p=0.725) between the Table Mountain and Narawntapu wild populations and the Bridport free-range captive population (33.3 – 50.0%). There was no significant difference ($p = 0.676$) in *Cryptosporidium* prevalence between the Freycinet, Devil Ark, Healesville, Cressy, Monarto, Taronga, and Western Plains Zoos populations (0.0 – 15.0%). However, the 37.5% prevalence of *Cryptosporidium* in the Bridport free-range population was significantly higher than the prevalence in the other captive populations ($p = 0.002$).

PCR screening identified 8/190 samples as positive for *Giardia.* There was no significant difference ($p = 1.000$) in the prevalence of *Giardia* between the intensive captive (0.0%) and free-range captive population types (1.6%). However, the prevalence in the wild population type (24.1%) was significantly higher than the captive population types ($p < 0.001$). At the population level, there was no significant difference in *Giardia* prevalence ($p = 1.000$) between the Table Mountain and Narawntapu wild populations (8.3 – 25.0%). Likewise, there was no significant difference in prevalence ($p = 0.262$) between any of the captive populations (0.0 – 8.3%).

3.2. Parasite prevalence in released vaccine-trial devils

3.2.1. Pre-release samples

Four of the six "types" of parasite described above were found in the 13 samples collected prior to the release of the vaccine-trial devils into the wild: *B. tasmaniensis*, the taeniid species, coccidia, and *Cryptosporidium*. The prevalence of *B. tasmaniensis* in the pre-release devils (46.2%; 95% C.I. = 20.4 – 73.9%) was not significantly different from the prevalence found in the Table Mountain and Narawntapu wild populations, or the Cressy, Bridport, and Freycinet captive populations (p = 0.344). The prevalence of the taeniid species in the pre-release samples (23.1%; 95% C.I. = 6.2 – 54.0%) was significantly higher (p = 0.034) than the prevalence of this parasite in all of the other population types. The prevalence of coccidia in the pre-release samples (7.7%; 95% C.I. = 0.4 – 37.9%) was not significantly different (p = 1) from the prevalence of coccidia in the Narawntapu, Bridport, Freycinet, Healesville, Monarto, and Western Plains Zoo populations. Likewise, the prevalence of *Cryptosporidium* in the pre-release sample (7.7%; 95% C.I. = 0.4 – 37.9%)) was not significantly different (P=1) from the prevalence of *Cryptosporidium* in the Freycinet, Devil Ark, Healesville, Cressy, Monarto, Taronga, and Western Plains Zoo populations.

3.2.2. Post-release samples

Only eight samples were collected following the release of the vaccine-trial devils into the wild. These eight samples came from four individual devils, three of which did not provide a pre-release sample. These devils will be identified as D1-4. D1 provided a pre-release sample and post-release sample 8 and 12 weeks following release; the pre-release sample was not found to contain any parasites, while the 8 and 12 weeks post-release samples both contained *B. tasmaniensis* only. D2 provided post-release samples 2, 4 and 8 weeks following release; the 2 weeks post-release sample was not found to contain any parasites, the 4 weeks post-release contained *Cryptosporidium* only, and the 8 weeks post-release sample was not found to contain any parasites. D3 provided post-release samples 2 and 4 weeks following release; neither sample was found to contain any parasites. D4 provided a single sample 4 weeks following release; this sample was found to contain *Cryptosporidium* only.

Table 2

Tasmanian devil parasite prevalence data; 95% confidence intervals are shown in square brackets for each population and population type. Four parasite types (*B. tasmaniensis*, a taeniid species, coccidia, and a strongylid species) were identified by faecal flotation and microscopy. Two further parasites (*Cryptosporidium* and *Giardia*) were detected using PCR. For each column, superscripted letters of the same type indicate that the prevalence data for the relevant groups were not statistically different from each other ($\alpha = 0.05$). Where there is no superscripted letter, the relevant group is significantly different from all other prevalence data in that column.

Population type	Facility/Location	*Baylisascaris tasmaniensis*	*Taeniid spp.*	*Coccidia*	*Strongylid spp.*	*Cryptosporidium*	*Giardia*
Wild	Narawntapu	33.3%^{a} [15.5, 56.9]	9.5%^{a} [1.7, 31.8]	0.0%^{a} [0.0, 19.2]	0.0%^{a} [0.0, 19.2]	33.3%^{a} [15.5, 56.9]	23.8%^{a} [9.1, 47.5]
	Table Mountain	37.5%^{a} [10.2, 74.1]	12.5%^{a} [0.66, 53.3]	25.0%^{b} [4.5, 64.4]	0.0%^{a} [0.0, 40.2]	50.0%^{a} [21.5, 78.5]	25.0%^{a} [4.5, 64.4]
	Wild (total)	**34.5% [18.6, 54.3]**	**10.3%^{b} [2.7, 28.5]**	**6.9% [1.2, 24.2]**	**0.0%^{cd} [0.0, 14.6]**	**37.9% [21.3, 57.6]**	**24.1% [11.0, 43.9]**
Intensive captive	Taronga	0.0%^{b} [0.0, 69.0]	0.0%^{a} [0.0, 69.0]	33.3%^{b} [1.8, 87.5]	0.0%^{a} [0.0, 69.0]	0.0%^{b} [0.0, 69.0]	0.0%^{b} [0.0, 69.0]
	Monarto	0.0%^{b} [0.0, 43.9]	0.0%^{a} [0.0, 43.9]	0.0%^{a} [0.0, 43.9]	0.0%^{a} [0.0, 43.9]	0.0%^{b} [0.0, 43.9]	0.0%^{b} [0.0, 43.9]
	Healesville	0.0%^{b} [0.0, 20.0]	5.0%^{a} [0.3, 26.9]	15.0%^{a} [4.0, 38.9]	5.0%^{b} [0.3, 26.9]	15.0%^{b} [4.0, 38.9]	0.0%^{b} [0.0, 20.0]
	Cressy	33.3%^{a} [14.4, 58.9]	0.0%^{a} [0.0, 21.9]	38.8%^{b} [18.3, 63.9]	16.7%^{b} [4.4, 42.3]	11.0%^{b} [1.9, 36.1]	0.0%^{b} [0.0, 21.9]
	Western Plains	0.0%^{b} [0.0, 34.5]	0.0%^{a} [0.0, 34.5]	10.0%^{a} [0.5, 45.9]	0.0%^{a} [0.0, 34.5]	0.0%^{b} [0.0, 34.5]	0.0%^{b} [0.0, 34.5]
	Intensive captive (total)	**12.1%^{c} [5.4, 23.9]**	**1.7%^{b} [0.1, 10.5]**	**20.7%^{c} [11.6, 33.7]**	**6.9%^{de} [2.2, 17.5]**	**8.6%^{c} [3.2, 19.7]**	**0.0%^{c} [0.0, 7.7]**
Free-range captive	Devil Ark	0.0%^{b} [0.0, 12.0]	0.0%^{a} [0.0, 12.0]	33.3%^{b} [19.1, 51.1]	0.0%^{a} [0.0, 12.0]	5.6%^{b} [1.0, 20.0]	0.0%^{b} [0.0, 12.0]
	Freycinet	33.3%^{a} [11.3, 64.6]	8.3%^{a} [0.43, 40.2]	16.7%^{a} [2.9, 49.1]	0.0%^{a} [0.0, 30.1]	0.0%^{b} [0.0, 30.1]	8.3%^{b} [0.43, 40.2]
	Bridport	18.8%^{a} [5.0, 46.3]	12.5%^{a} [2.2, 39.6]	12.5%^{a} [2.2, 39.6]	0.0%^{a} [0.0, 24.1]	37.5%^{a} [16.3, 64.1]	0.0%^{b} [0.0, 24.1]
	Free-range captive (total)	**10.9%^{c} [4.9, 21.8]**	**4.7%^{b} [1.2, 14.0]**	**25.0%^{c} [15.4, 37.7]**	**0.0%^{ce} [0.0, 7.1]**	**12.5%^{c} [5.9, 23.7]**	**1.6%^{c} [0.0, 9.5]**

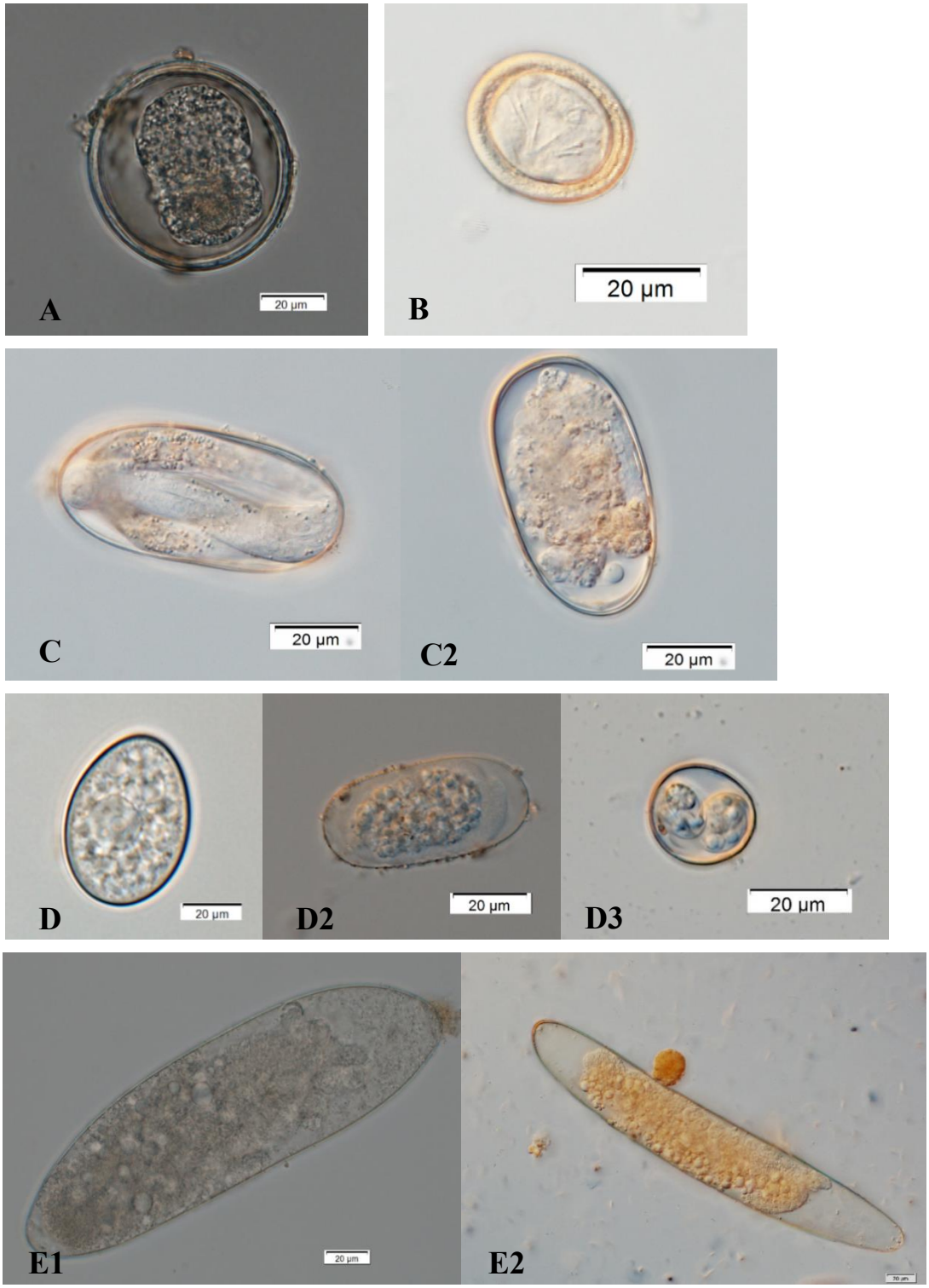

Figure 1

Parasites of the Tasmanian devil. Parasite stages were digitally photographed and measured at 400X magnification under a BX53 microscope equipped with DIC optics and CellSens interface software (Olympus, Shinjuku, Japan). Measure bars in all photographs are 20 µm. A = *B. tasmaniensis* eggs, B = taeniid species eggs, presumed to be *A. dasyuri*; C1 and C2 = strongylid species eggs, thought to be *W. sarcophili*, larvated and non-larvated eggs; D1, D2, and D3 = coccidial oocysts of unknown species, D3 is sporulated and belongs to genus *Isospora;* E1 and E2 = mite eggs of unknown species.

3.3. Parasite diversity and distribution

Faecal samples were found to have between zero and three types of parasite (Table 3, Figure 2). The distribution of infections and coinfections were compared between the different Tasmanian devil population types and within the different populations using the Kruskal-Wallis rank sum test. Overall, 51.0% of samples had zero parasites, 39.1% of samples had one type of parasite, 7.3% of samples had two types of parasites, and 2.6% of samples had three types of parasites. No significant difference was found in the distribution of infections between the intensive and free-range captive population types ($p = 0.711$). However, there was a significant difference between wild and captive population types ($p < 0.001$). In the wild population type, 75.9% of samples had at least one parasite, while in the captive population type only 42.6% of samples had at least one parasite. Co-infections – that is, concurrent infections with two or more types of parasite – were more common in wild devils compared with captive devils. Altogether, 22/190 samples were co-infected (Table 4). The two most common parasites found in co-infected samples were *B. tasmaniensis* (68.2% of co-infected samples), and *Cryptosporidium* (50.0% of co-infected samples), however, co-infection distributions varied between population types. *B. tasmaniensis* was present in 72.7% of co-infected wild samples, but only 55.5% of co-infected captive samples. *Cryptosporidium* was present in 45.5% of co-infected wild samples, but only 33.3% of co-infected captive samples. Conversely, coccidia were present in 66.7% of co-infected captive samples, but only 18.2% of co-infected wild samples.

At the population level, there was no significant difference in the distribution of infections between the Narawntapu and Table Mountain wild populations ($p = 0.096$). When analysed, the captive populations fell into three groups with significantly different parasite distributions. There was no significant difference between the Freycinet, Bridport, Cressy, and Healesville captive populations, which were made up of a mixture of uninfected, singly-infected, and co-infected devils (group 1; $p = 0.112$). Likewise, there was no significant difference between the Monarto, and Western Plains Zoo captive populations, which were composed almost entirely of uninfected devils (group 2; $p = 0.403$). The distribution of infections in the Devil Ark and Taronga captive populationswere not significantly differently from each other (group 3; $p = 0.851$) and consisted of uninfected and singly-infected devils,. Groups 1, 2 and 3 were significantly different ($p < 0.001$).

Table 3

Tasmanian devil parasite diversity and distribution. Number of samples per population type and facility with zero, one, two, or three different types of parasites. The percentage of the population with *x* of samples is given in brackets. For the 'Mean' column, the superscripted letters indicate that the prevalence data for populations with the same letter were not statistically different (α=0.05).

Population type	**Facility/Location**	**Number of samples with *x* parasites in each population type and facility**				**Total**	**Mean**
		***x* = 0**	***x* = 1**	***x* = 2**	***x* = 3**		
Wild	Narawntapu	7	9	3	2	21	1^{a}
	Table Mountain	0	4	4	0	8	1.5^{a}
	Wild total	**7**	**13**	**7**	**2**	**29**	**1.14^{j}**
Intensive captive	Taronga	2	1	0	0	3	0.33^{d}
	Monarto	7	0	0	0	7	0^{c}
	Healesville	13	6	0	1	20	0.45^{b}
	Cressy	3	14	1	0	18	0.89^{b}
	Western Plains	9	1	0	0	10	0.1^{c}
	Intensive captive total	**34**	**22**	**1**	**1**	**58**	**0.47^{k}**
Free-range captive	Devil Ark	22	14	0	0	36	0.39^{d}
	Freycinet	5	6	1	0	12	0.58^{b}
	Bridport	9	4	2	1	16	0.69^{b}
	Free-range captive total	**36**	**24**	**3**	**1**	**64**	**0.52^{k}**
	Total	**77**	**59**	**11**	**4**	**151**	**0.62**

Table 4

Co-infection distributions for Tasmanian devil parasites. Altogether, 22/190 samples were concurrently infected by two or more parasite types.

	B + Cr	**B + Co**	**B + T**	**B + T + Co**	**B + G**	**B + Cr + T**	**Cr + Co**	**Cr + G**	**T + Co**
Wild	3	1	1	0	2	1	1	2	0
Intensive captive	1	1	0	1	0	0	1	0	0
Free-range captive	1	0	0	0	0	1	0	0	3
Pre-release	0	1	1	0	0	0	0	0	0

B = *B. tasmaniensis*, Cr = *Cryptosporidium*, Co = coccidia, T = taeniid spp., G = *Giardia*.

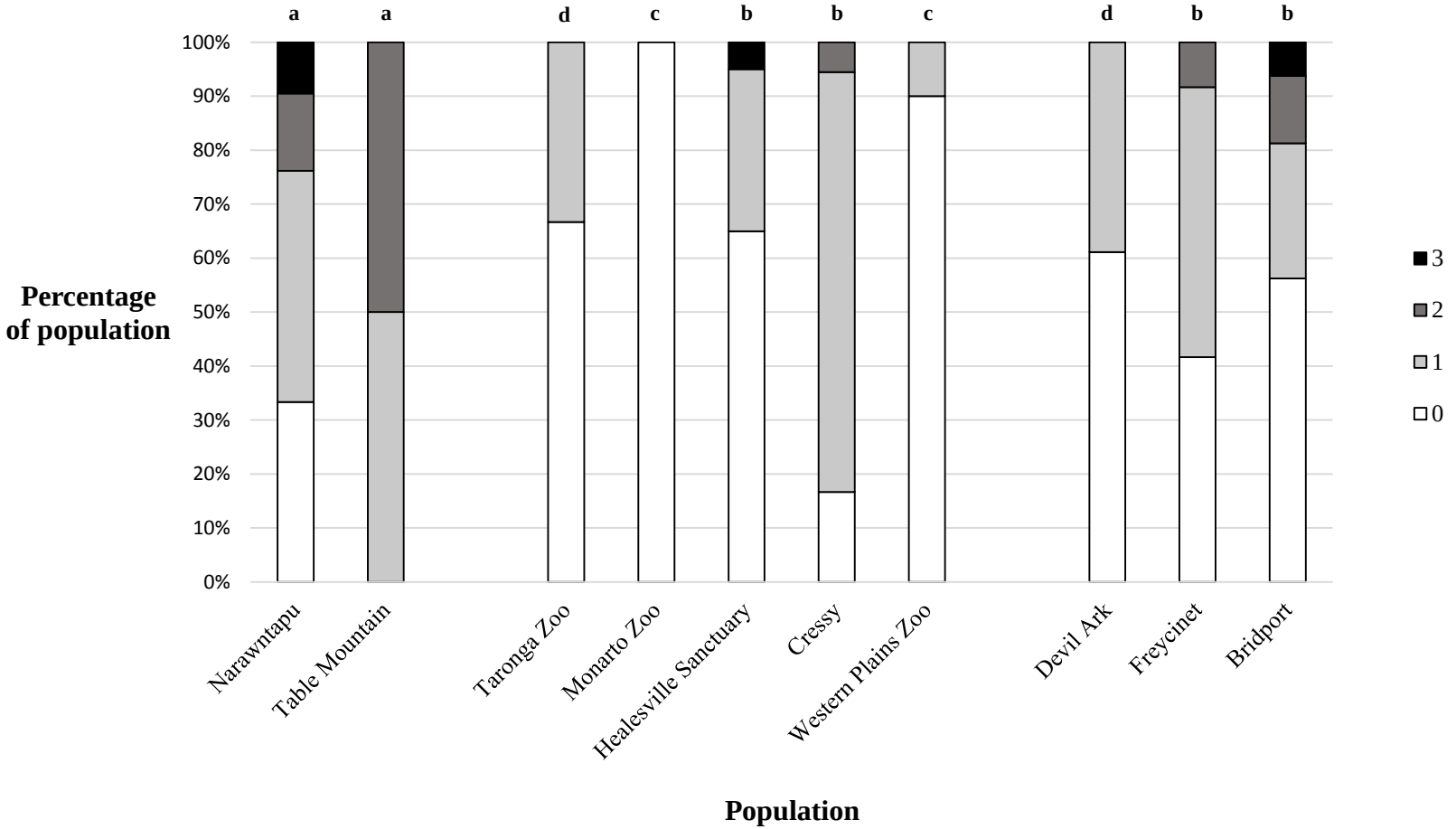

Figure 2

Distribution of Tasmanian devil parasites within different populations. Six "types" of parasite were identified, and samples were found to have between zero and three different types of parasite. This graph shows the proportion of each population made up of samples containing zero, one, two, or three types of parasite. Letters above the columns (a,b,c,d) indicate statistical significance; the distribution of parasites within columns with the same letter are not significantly different (Kruskal-wallis, $P<0.05$).

4. Discussion

This study found significant differences in the prevalence of gastrointestinal parasites between captive and wild populations of Tasmanian devils. *B. tasmaniensis*, *Cryptosporidium* and *Giardia* were all significantly more prevalent in wild compared with captive devils. Conversely, coccidia and the strongylid species were both more prevalent in captive compared with wild devils. There was no significant different in the prevalence of a taeniid species between wild and captive populations, but this parasite had a significantly higher prevalence in pre-release samples obtained from captive devils that were subsequently released into the wild. More than 70.0% of wild devils were infected with at least one parasite type, and more than 30.0% were co-infected with two or more parasite types. In contrast, less than 50.0% of captive devils were infected by parasites, and less than 10.0% were co-infected.

There are several mutually possible explanations for the differences seen in parasite prevalence and diversity between the different devil populations. The relative abundance of food in captivity might be a factor in the higher prevalence of certain parasites in wild compared with captive devils. While captive devils are maintained on diets aimed at keeping them in ideal condition, wild devils experience fluctuations in food availability, and face the added energy cost of having to travel and compete for food and other resources. The lower nutritional status experienced by wild devils may result in less resources being allocated to immune function than is ideal (Zuk and Stoehr, 2002), allowing for more frequent parasite infections in wild compared with captive devils.

Anthelmintic treatment of captive devils could also explain the lower prevalence of certain parasites, specifically *B. tasmaniensis*, in captive devils: one of the captive populations of devils is treated routinely with anthelmintics (Devil Ark, pers. comm. Dean Reid), while the other captive populations are only treated as required (pers. comm. Anthony Brett-Lewis, Steve Kleinig, Althea Guinsberg, Kathy Starr, and Olivia Barnard).

Certain Tasmanian devil parasites, for example *B. tasmaniensis* and *A. dasyuri*, have complex life-cycles with life stages in different host species, thus requiring contact between these multiple host species for transmission. These lifecycles are far less likely to be completed in captive devils with restricted access to the other requisite host species. Even for parasites with direct lifecycles, captive environments may be less conducive to parasite transmission due to the cleaning of enclosures and removal of faecal material, and in some cases captive devils are housed individually which would prevent transmission between individuals. However, this study did find a higher prevalence of coccidia in captive compared with wild devils; this could be due to stress and impaired immunity associated with captivity, or to captive devils being housed at higher densities than occur in the wild (Cunningham, 1996). If devils are treated with anthelmintics when brought in

from the wild, and are subsequently maintained in an environment free from infective parasite stages, then they will remain parasite-free without continued anthelmintic treatment. Similarly, if captive-bred devils are never exposed to infective parasite stages, they will remain parasite-free.

This study found *B. tasmaniensis* to be significantly more prevalent in wild compared with captive devils. In a comparison of the captive populations, *B. tasmaniensis* was significantly more prevalent in Tasmanian compared with mainland Australian captive populations; in fact, this nematode was not found in any populations on mainland Australia. The 46.2% prevalence of *B. tasmaniensis* in pre-release samples collected from vaccine-trial devils was higher than the prevalence in any other group, though not statistically different from the prevalence in the wild populations, or the Cressy, Bridport, and Freycinet captive populations. The vaccine-trial devils were housed in the Freycinet and TasZoo free-range enclosures for several months prior to sampling, so it is not surprising that their prevalence is similar to that seen in devils housed in Tasmanian free-range facilities.

Taeniid eggs were identified in faecal samples from both wild and captive devils, and there was no significant difference between population types. The taeniid species identified here is likely to represent *A. dasyuri*, though there is a slight possibility that it could be *D. robusta*. Unfortunately, there are no morphological guidelines for the differentiation of *A. dasyuri* and *D. robusta* eggs (pers. comm. Ian Beveridge). However, these eggs are much more likely to be *A. dasyuri* eggs as this species has been historically reported to infect a large proportion of devils (Gregory et al., 1975), while *D. robusta* is classified as Rare under the Tasmanian Threatened Species Protection Act, 1995. The 10.3% wild prevalence found in this study was far lower than the 99.7% prevalence reported for *A. dasyuri* in 1975 (Gregory et al., 1975). Decreases in parasite prevalence do occur as a result of decreased host density (Arneberg et al., 1998), as has occurred with Tasmanian devils due to DFTD (Bender et al., 2014). However, the prevalence found in this study may also to be an underestimate due to the lower sensitivity of faecal flotations compared with post-mortems, which have been the primary method of studying Tasmanian devil parasites in the past (Overgaauw, 1997).

Interestingly, only one devil in a facility on mainland Australia was infected by this taeniid, and this devil had been translocated from a Tasmanian facility less than two months before a sample was collected (pers. comm. Kathy Starr), making it likely that this devil was infected in Tasmania. Additionally, taeniid eggs were significantly more prevalent in pre-release samples obtained from the vaccine trial devils compared with all other populations. The reason for this is unknown; it could be due to the low sample size, or it could be stress-related as vaccine trial devils would have undergone more handling compared to other free-range devils. Alternatively, it could be due to

some interaction between the DFTD-vaccine and the devils' immune systems resulting in these devils being more susceptible to helminth infection. Interactions between vaccines and helminths are known to occur; studies have shown vaccinations to result in less protective immune responses in helminth-infected compared with uninfected animals (Bobat et al., 2014; Elias et al., 2005), but no studies have investigated whether vaccination could potentially increase susceptibility to non-target parasites.

Coccidial oocysts were identified in faecal samples from both wild and captive Tasmanian devils. Coccidia (Apicomplexa: Coccidia) are a group of obligate intracellular protozoan parasites with direct lifecycles (Bandoni and Duszynski, 1988). Coccidia have never been reported in devils in the literature; rather, their absence has been noted (Munday, 1988). However routine faecal flotations of wild devils as part of the save the Tasmanian devil Program have detected *Eimeria* oocysts (pers.comm. Sarah Peck). This study identified at least three different types of coccidia; though the exact species of coccidia could not be determined, some sporulated coccidial oocysts were identified as belonging to the genus *Isospora* based on the presence of exactly two sporocysts.

Coccidia were significantly more prevalent in captive compared with wild devils. This higher prevalence of coccidia in captive compared with wild populations of devils, combined with the fact that coccidia have never before been officially reported in devils, could indicate that these coccidia are not Tasmanian devil parasites. It is possible that these oocysts do not represent an infection at all, but are pseudoparasites passively passing through the guts of these devils as a result of ingestion of oocysts (Gressler et al., 2009). If it turns out that these coccidia are infecting Tasmanian devils, it is possible that they represent spill-over of coccidia from humans or domestic animals. Further work will be needed in order to identify the species of coccidia present. If the coccidia are found to represent a devil species, the higher prevalence seen in captive devils could be due to stress and impaired immunity associated with captivity (Cunningham, 1996).

The strongylid eggs found in this study are thought to represent *W. sarcophili*, a species of strongylid nematode known from Tasmanian devils (Humphery-Smith and Durette-Desset, 1981). Strongylid eggs were detected in only four samples, all from intensive captive facilities: three from Cressy and one from Healesville. A 1981 study estimated the prevalence of *W. sarcophili* as 8.0% (n=25), based on post mortems (Humphery-Smith and Durette-Desset, 1981). Such a low historical prevalence, combined with the use of faecal flotation for detection rather than the more sensitive post mortems, could account for this study not detecting this nematode in wild devils. It is possible that wild devils were infected, but at a low intensity such that eggs were not detected. Conversely, the finding of this nematode in intensively managed devils could indicate that captive devils

harbour higher parasite burdens, possibly due to stress and crowding associated with captivity (Cunningham, 1996).

PCR analysis identified *Cryptosporidium* in both wild and captive devils, though this parasite was significantly more prevalent in wild devils. This is the first report of *Cryptosporidium* from Tasmanian devils. PCR is the most common method of detection for *Cryptosporidium*, and has the added benefit of allowing for species identification (Morgan and Thompson, 1998). Two juvenile devils from the Bridport free-range captive population tested positive for *Cryptosporidium* on two occasions, 21 days apart. Sequencing of these samples (Chapter 2) indicated that, for both devils, the *Cryptosporidium* detected in samples at both times were genetically identical. Additionally, one devil in the group of released devils (D2) was positive for *Cryptosporidium* when sampled four weeks post-release, but was negative for *Cryptosporidium* eight weeks post-release. Together, these findings could indicate that *Cryptosporidium* infections in devils last at least 21 days, but possibly not as long as 28 days.

PCR analysis found *Giardia* to be significantly more prevalent in wild compared with captive devils. The prevalence of *Giardia* in wild devils was estimated as 24.1%; this is higher than previous estimates of 8.3% (n = 12) and 6.0% (n = 32) (Davies, 1995; Kettlewell et al., 1998). This higher estimate is likely due to the fact that the molecular techniques used here are far more sensitive than the faecal flotation methods used in prior studies of *Giardia* in Tasmanian devils (Morgan et al., 1998; McGlade et. al., 2003).

Zinc sulfate faecal flotations were used as the main detection method in this study as it was thought that this procedure would allow detection of the largest range of different gastrointestinal parasites. The findings here suggest that parasite prevalence and diversity differ between captive and wild populations of devils, and future studies will build on these findings. It will be interesting to see if the patterns seen here hold true for other parasite species. Future studies should perform tests targeting parasite types that were not targeted by this study; for example, sedimentation tests would enable detection of the three known species of devil digenea: *Mehlisia acuminata*, *Neodiplostomum sarcophila* and *N. diaboli* (Spratt et al., 1991). Future studies could also investigate how parasite abundance differs between different devils and between captive and wild populations by performing faecal egg/oocyst counts on samples from different devils.

This study aimed to investigate whether changes in parasite prevalence might occur when captive devils are released into the wild, but the small number of post-release samples collected meant that this was not possible; if captive devils continue to be released, it would be valuable to monitor these potential changes. It would also be of value to investigate the potential effects of DFTD on devil parasites; in this study, 2/190 samples came from a DFTD-infected devil, and no

significant difference was seen in parasite prevalence for these samples (data not shown), but a broader investigation is warranted.

Identification of helminth eggs in this project was made difficult by the incomplete representations given of Tasmanian devil parasites in historical accounts. Most past studies on devil parasites have focussed on describing adult helminths rather than egg morphology (Dubois and Angel, 1972; Johnston, 1912; Johnston and Mawson, 1940; Sandars, 1957). The photographs taken as part of this study (Figure 1) should aid future identification of Tasmanian devil gastrointestinal parasites.

Estimates of prevalence in this study, aside from those for *Cryptosporidium* and *Giardia*, are likely to be lower than true prevalence due to the lower sensitivity of faecal flotation compared with molecular techniques and post mortems (Overgaauw, 1997). Ideally, the results of non-invasive studies such as this would be supplemented by post mortems of devils lost in the captive and wild populations. Future work should combine morphological information on different life stages – adult helminths, eggs, larvae –with molecular data to provide a full picture of each parasite.

5. Conclusions

This study aimed to provide baseline data on the prevalence and diversity of Tasmanian devil gastrointestinal parasites, and to examine the possibility that conservation management may be changing devil parasite-communities. Prevalence estimates for six gastrointestinal parasite types found significant differences for most of these parasites between captive and wild populations of Tasmanian devils. Additionally, wild devils were significantly more likely to be infected by at least one type of parasite. These findings suggest that conservation management may be changing host-parasite interactions in the Tasmanian devil. Though only preliminary, these results will help guide the Save the Tasmanian Devil program in their goal to conserve symbionts and parasites associated with Tasmanian devils.

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